University of Alberta

Olfactory and Behavioural Responses to Putative Steroidal Pheromones in the Round Goby, *Neogobius melanostomus*

by

Cheryl Anne Murphy ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Olfactory and Behavioural Responses to Putative Steroidal Pheromones in the Round Goby, *Neogobius melanostomus* submitted by Cheryl Anne Murphy in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Cell Biology.

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This thesis is dedicated to
Janet and Peter Murphy

ABSTRACT

To identify potential hormonal pheromones of the round goby (*Neogobius melanostomus*), a species recently introduced to the Great Lakes, I used electro-olfactogram (EOG) recording to examine olfactory responsiveness to approximately 150 steroids and prostaglandins. *Neogobius* detects free and conjugated 18-, 19- and 21-carbon steroids, but no prostaglandins. EOG cross-adaptation, used to determine if *Neogobius* can discriminate the detected compounds at the sensory level, suggested that the detected steroids act via four classes of olfactory receptor mechanisms named according to the most potent ligand for each: estrone, 17ß-estradiol-3-glucuronide, etiocholanolone, and dehydroepiandrosterone-3-sulphate. Although none of the detected steroids induced reproductive behaviours, exposure to steroids from three of the four receptor classes (estrone, estradiol-glucuronide, or etiocholanolone) increased ventilation rate in males, whereas only etiocholanolone increased ventilation in females. The estrone- and estradiol-glucuronide-induced ventilation increase in males appears to be androgen-dependent because, in females, androgen implants induced the ventilatory response to these steroids within two weeks. Using the ventilation increase as a behavioral bioassay of steroid detection, behavioral cross-adaptation studies in males demonstrated that steroids discriminated at the sensory level are also discriminated behaviourally. These findings, the first olfactory study of hormonal pheromones in a perciform species, suggest the round goby has evolved a complex steroidal pheromone system.

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TABLE OF CONTENTS

Chapter		Page
1.	INTRODUCTION	1
2.	FISH	15
3.	OLFACTORY RESPONSE TO PUTATIVE STEROIDAL PHEROMONES	16
	3.1. Methods	16
	3.1.1. Electro-olfactogram (EOG) procedure	16
	3.1.2. Tested odours	18
	3.1.3. EOG concentration response studies	20
	3.1.4. EOG cross-adaptation	21
	3.2. Results	25
	3.2.1. Response to test odours	25
	3.2.2. EOG concentration response results	26
	3.2.3. EOG cross-adaptation results	26
4.	BEHAVIOURAL RESPONSE TO PUTATIVE STEROIDAL PHEROMONES	52
	4.1. Methods	52
	4.1.1. Behavioural repertoire	52
	4.1.1.1. Spontaneous behaviours observed in the laboratory	52
	4.1.1.2. Behaviours of potential reproductive significance	53
	4.1.1.3. Feeding behaviours	55
	4.1.1.4. Behavioural measures of activity	56
	4.1.2. Behavioural responses to detected steroids	56
	4.1.2.1. Testing apparatus	57
	4.1.2.2. Testing procedure	57
	4.1.2.3. Test odours	58
	4.1.2.4. Data analysis	59
	4.1.3. Behavioural concentration response studies	60
	4.1.4. Behavioural cross-adaptation	61
	4.2. Results	63
	4.2.1. Behavioural responses to detected steroids	63
	4.2.2. Behavioural concentration response studies	64
	4.2.3. Behavioural cross-adaptation	65
5.	BEHAVIOURAL MASCULINIZATION OF FEMALES WITH METHYLTESTOSTERONE (MT)	94
	5.1. Methods	94
	5.2. Results	96

TABLE OF CONTENTS (cont.)

Chapter		Page
6	Discussion	101
	6.1. Olfactory responses to steroids and steroid metabolites	102
	6.1.1. Cross-adaptation	102
	6.1.2. Proposed classes of olfactory receptors	105
	6.1.2.1. Estrone (E1) receptor	105
	6.1.2.2. Estradiol (E2-3g) receptor	106
	6.1.2.3. Dehydroepiandrosterone-sulphate (DHEA-s) receptor	107
	6.1.2.4. Etiocholanolone (Etio) receptor	108
	6.1.3. Olfactory insensitivity to prostaglandins	109
	6.1.4. Olfactory insensitivity to maturation inducing steroids	110
	6.2. Behaviour	111
	6.2.1. Additional cues	111
	6.2.2. Chemical mixtures	112
	6.2.3. Pheromones with priming effects	114
	6.2.4. Inappropriate reproductive condition	115
	6.2.5. Ventilation behaviour	115
	6.2.6. Relationship between EOG and behavioural responses	117
	6.2.7. Possible pheromonal function of detected steroids	118
	6.2.7.1. C18 steroids (estrogens and their metabolites)	119
	6.2.7.2. C19 and C21 steroids	120
	6.2.7.3. DHEA-s	121
	6.2.7.4. Steroidal sex pheromones of the round goby	121
	6.3. Summary	124
7.	LITERATURE CITED	125
APPENDIX A.	List of Steroids and Prostaglandins tested in EOG	137
APPENDIX B.	Spectrograph of Gaping behaviour	141
APPENDIX C.	Spectrograph of Coughing behaviour	142
APPENDIX D.	Structure of steroids detected by the four receptor mechanisms	143

LIST OF TABLES

Table		Page
3.1.	Detected steroids, grouped according to chemical structure with EOG responses	30-31
3.2.	Detected steroids, grouped according to receptor class	32-34
3.3.	Detected steroids that act via two receptor mechanisms	35
4.1.	Duration of ventilation rate response of male and female gobies in response to steroids	67

LIST OF FIGURES

Figure		Page
3.1.	EOG responses	37
3.2.	Comparison of male and female EOG responses	39
3.3.	Concentration response profiles for selected steroids	41
3.4.	Typical EOG concentration response profile	43
3.5.	EOG response to 2-sec steroid pulses delivered 30 min apart	45
3.6.	Effect of adaptation to E1 on EOG response to E2 and E2-3g	47
3.7.	EOG responses to steroids before and during adaptation to E1, E2-3g, and DHEA-g	49
3.8.	EOG responses to steroids before and during adaptation to Etio and DHEA-s	51
4.1.	Ventilation rate response of male and female gobies to EtOH and T-g	69
4.2.	Ventilation rate response of male and female gobies to E1 and E2-3g	71
4.3.	Ventilation rate response of male and female gobies to Etio, Etio-g and Andr-s	73
4.4.	Ventilation rate response of male and female gobies to DHEA-s, DHEA-g and male response to steroid mixture	75
4.5.	Ventilation rate response of male and female gobies to L-alanine and food odour	77
4.6.	Eating frequency response of gobies to food odour, L-alanine, and EtOH	79
4.7.	Ventilation rate response of male gobies to increasing concentrations of E1, E2-3g, and Etio	81

LIST OF FIGURES (cont)

Figure		Page
4.8.	Prestimulus ventilation rates in comparison to post-stimulus ventilation rates in male gobies	83
4.9.	Ventilation rate response of male gobies to 10^{-9} M Andr-s and E2	85
4.10.	Ventilation rate response of male gobies to E1 and sequential 10^{-9} M steroids	87
4.11.	Ventilation rate response of male gobies to Etio and sequential 10^{-9} M steroids	89
4.12.	Ventilation rate response of male gobies to DHEA-s and sequential 10^{-9} M Andr-s	91
4.13.	Hopping frequency of male gobies in response to E1 and sequential 10^{-9} M steroids	93
5.1.	Ventilation rate response of implanted (MT and Blank) female gobies to sequential steroids	98
5.2.	Anesthetized implanted (MT and Blank) female gobies	100

1. INTRODUCTION

The proposal that in aquatic organisms, released hormones and hormone metabolites can function as pheromones was first suggested by Kitteredge *et al.* (1971), who observed that female crabs must be inseminated soon after moulting, and implied that males respond to the release of the moulting hormone, crustecdysone, as an indicator of mating opportunities. Upon detection of crustecdysone, males change behaviour patterns, and perform pre-copulatory behaviour (Kitteredge *et al*, 1971). Kitteredge *et al.* (1971) extended the crab model to aquatic organisms in general, suggesting that it is probable that many aquatic organisms have evolved pheromonal function for released hormones and hormone metabolites, due to the likelihood of the chance development of a receptor for these compounds in olfactory neurons. Reproductive behaviour or physiology of the pheromone receiver can then be synchronized with a particular physiological event in conspecifics, increasing the chance of reproductive success. After Doving's (1976) suggestion that this hypothesis be extended to fish, numerous advances in teleost pheromone research indicate that hormonally derived chemical signals (*hormonal pheromones*) are also used by many fish (Stacey and Sorensen, 1991; Sorensen, 1992; Stacey *et al.* 1994).

Karlsson and Lüscher (1959) first defined pheromones as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process". In teleost fish, many examples have been documented where an

identified water-borne hormone, or hormone metabolite, acting through the olfactory system, evokes a specific change in behaviour or physiology, qualifying these compounds as pheromones. Knowledge of the nature and function of external chemical compounds that are detected by the olfactory system, and that can induce behavioural and physiological changes in an organism, is a powerful tool that can be applied to many fish culture and management practices. In particular, pheromone manipulation of fish behaviour and physiology may be a practical method to control exotic pest species. Pheromonal methods may be less expensive and labour intensive, and more direct and species-specific, than traditional methods that involve piscicides and male sterilizations (see Wiley and Wydoski, 1993 for review of piscicides; Hanson and Manion, 1980 for sterilization of sea lamprey, *Petromyzon marinus*).

The objective of this study was to identify hormonal pheromones of the round goby, *Neogobius melanostomus*, which was introduced to the Great Lakes from the Black and Caspian Seas in ships' ballast (Crossman *et al.*, 1992; Jude *et al.*, 1992). Increasing numbers of the round goby might have contributed to the decline of native mottled sculpins (*Cottus bairdi*) and other benthic fishes (Jude *et al.* 1995; Dubs and Corkum, 1996).

Round goby impact:

Round gobies are aggressive, easily take over optimal spawning habitats of native benthic species, and eat eggs and young of other fishes (Dubs and Corkum, 1996; Charlebois *et al.*, 1997). Since round gobies can spawn several times in a season, and

have mean estimated fecundities ranging from 252-1818 eggs (MacInnis and Corkum, in press), they can reproductively outcompete single spawners like the mottled sculpin, *Cottus bairdi*, a species with a mean fecundity of 257 eggs (Bailey, 1952). Additionally, because round gobies eat zebra mussels (*Dreissena polymorpha*) that can accumulate polychlorinated biphenyls, and in turn are preyed upon by sport fish, they might facilitate introduction of toxins into the food chain (Ray and Corkum, 1997). In reality, little is known about how the round goby will ultimately affect the Great Lakes ecosystem, but with rapidly increasing numbers, there appears to be a potential for serious impact. The round goby has now been reported to have established thriving populations in all of the Great Lakes and is close to invading the Mississippi River drainage system, raising concern over potential detrimental effects on many North American native fish species and ecosystems (Charlebois *et al.*, 1997). This study was undertaken on the premise that an understanding of goby pheromones might lead to methods for control or eradication of this species in the Great Lakes. Pheromonal techniques have been used successfully to control and monitor a variety of insect pests (Niwa *et al.*, 1988; Deland *et al.*, 1994; Pree *et al.*, 1994; Cork and Basu, 1996; Cork *et al.*, 1996; Shorey and Gerber, 1996), but I know of no studies that have used pheromones to manipulate fish under natural conditions.

Research approach:

The identification of hormonal compounds that might have pheromonal function in a fish requires information about hormonal synthesis, release, and detection, as well as

an understanding of the behavioural and/or physiological responses the detected compounds can evoke. Over the past 20 years, experiments addressing these four aspects of pheromone identification in goldfish *(Carassius auratus)* have produced the most detailed model of hormonal pheromones in a fish. To initiate investigation into the hormonal pheromone system of the round goby, I first attempted to determine which prostaglandins, steroids, and metabolites are detected by the olfactory system, and then determined if these compounds induce behavioural responses; research on goldfish and common carp (*Cyprinus carpio*) has shown a good correlation between olfactory response and biological response (Sorensen *et al.*, 1990; Irvine and Sorensen, 1993; Stacey *et al.*, 1994). To understand fully the pheromonal function(s) of the detected hormones identified in this study, further investigations are required to determine what detected hormones are released by male and female gobies, and when in the reproductive cycle the detected hormones are released. Also, a detailed description of the mating system of the round goby is critical to place the function of pheromones in biological context.

Mating strategies and hormonal pheromones in gobiids:

Casual observations of *Neogobius* suggest that this species uses a mating strategy that is typical of many gobiids (Breder and Rosen, 1966; Miller, 1984). Males migrate from deeper waters to spawning grounds in spring, set up territories prior to arrival of females, and defend a nest site to which females are attracted for spawning (Moiseyeva and Rudenko, 1979; Kotvun, 1980; Kulikova, 1985). Males care for eggs and appear to care for multiple clutches at the same time (MacInnis and Corkum, in press).

As with male *Bathygobius soporator* (Tavolga, 1956), male round gobies use visual displays (colouration changes and posturing) and acoustical signals when courting females (Protosov *et al.*, 1965; Moiseyeva and Rudenko, 1979; Kulikova, 1985). Although no study has examined the role of pheromones in the reproduction of round gobies, evidence from other gobiids suggest that hormonal pheromones play an important role in courtship.

Observations on the blind goby, *Typhlogobius californiensis,* revealed that this species exhibits agonistic behaviours only towards members of the same gender, and that gender recognition is dependent on chemoreception (MacGinitie, 1939). Additionally, classic work by Tavolga (1956) demonstrated that male *Bathygobius* will perform courtship behaviour when exposed to ovarian fluid from ovulated females. If males are rendered anosmic, they fail to respond to the ovarian fluid, indicating that the courtship response is mediated by a female chemical signal detected through olfaction.

Territorial male black gobies, *Gobius jozo* are purported to release a conjugated androgen, 5β-androstan-3α-ol-17-one-3-glucuronide (etiocholanolone-glucuronide; Etio-g) that attracts ovulated females to nest sites and stimulates oviposition (Colombo *et al.*, 1982). Etio-g is proposed to be produced in a mesorchial gland, a Leydig-cell rich accessory gland located next to the testis (Colombo and Burighel, 1974). The mesorchial gland is commonly found in gobiids (Miller, 1984), and appears to be one of the few examples of structural specialization associated with pheromonal function in fish. In *Neogobius* the mesorchial glands have been termed seminal vesicles (Soin and Chepurnov 1987).

In *G. jozo* the mesorchial glands synthesize large amounts of conjugated 5β-reduced androgens (like Etio-g) through a biosynthetic pathway that begins with the hydroxylation of pregnenolone: pregnenolone--- 17α-hydroxypregnenolone--- dehydroepiandrosterone --- androst-4-ene-3-17-dione--- 5β-androstan-3,17-dione--- etiocholanolone---- conjugated etiocholanolone (Colombo *et al.*, 1977). Colombo *et al.* (1980) propose that having specialized glands for pheromone synthesis allows for the production of larger quantities of a chemical signal, and such a specialization might have evolved as females exert sexual selection based on male signal strength. The presence of specialized glands for pheromonal synthesis suggests that some gobies have evolved chemical communication. In cases where chemical communication occurs, a specialized signal (such as that released by the mesorchial gland) issued by the sender is perceived by a receiver, and both sender and receiver benefit from the response of the receiver (Stacey and Sorensen, 1991). Such communication is proposed to evolve from '*chemical spying*'; in which only the receiver benefits, and in which the receiver's response is not a selective force for signal specialization (Stacey and Sorensen, 1991).

Fish pheromones:

Chemical compounds that act through the olfactory system have been categorized into four main classes of odours: bile acids, amino acids, sex steroids and their metabolites, and prostaglandins (Sorensen and Caprio, 1997). Bile acids, produced to emulsify ingested fats, are released to the external environment where they are proposed to function as social and migratory cues in some fish (Li and Sorensen, 1997; Sorensen

and Caprio, 1997). Amino acids, particularly L-amino acids, appear to be detected by all fish, and function as feeding cues (Hara, 1992). Sex steroids and prostaglandins can be collectively labelled as hormonal pheromones and function in reproductive signalling.

Hormonal pheromones and feeding stimuli appear to act through different olfactory mechanisms. There is evidence that hormonal pheromones are detected by microvillous receptor cells within the olfactory epithelium, whereas feeding cues are detected by ciliated receptors (Zippel *et al.*, 1997). Axon terminals of receptor cells for particular odours also appear to be spatially segregated within the olfactory bulb; those for amino acids are likely located in different regions than those for pheromones (Thommesen, 1982; Friedrich and Korsching, 1997). Also, hormonal pheromone binding to receptor cells stimulates the medial olfactory tract (Sorensen *et al.*, 1991), which projects to regions of the ventral telencephalon that have been suggested to control sexual behaviour (goldfish; Kyle and Peter, 1982: sockeye salmon, *Oncorhynchus nerka*; Satou *et al.*, 1984). In goldfish, sectioning the medial olfactory tract eliminates pheromonal effects on behaviour and physiology without affecting feeding responses to food odour, whereas sectioning the lateral olfactory tract inhibits feeding behaviours without affecting hormonal pheromone action (Stacey and Kyle, 1983; Dulka and Stacey, 1990).

Olfactory sensitivity to pheromones has been determined largely through the use of the electro-olfactogram procedure (EOG). In EOG recordings, an extracellular electrode is placed next to the surface of the olfactory epithelium, and measures the voltage change believed to represent summed olfactory receptor generator potentials produced in response to odours (Ottoson, 1971). After EOG recording has identified

what test compounds a species can detect, EOG cross-adaptation then can be used to determine if the olfactory system is capable of discriminating odours (Caprio and Byrd, 1984; Sorensen *et al*., 1995b; Li and Sorensen, 1997). In EOG cross-adaptation, the olfactory epithelium is exposed to one compound (the adapting compound) until adaptation has occurred, at which time the olfactory epithelium is also exposed to a second detected compound (the test compound). The rationale behind EOG cross-adaptation is that, if the EOG response to the test compound is unaffected by adaptation, it is detected by a different olfactory receptor mechanism than the adapting compound, whereas if the response is reduced or abolished by adaptation, it is operating via the same olfactory receptor mechanism as the adapting compound (Caprio and Byrd, 1984). As defined by Sorensen *et al* (1995b), *olfactory receptor mechanism* refers to the entire physiological entity responsible for a voltage change (recorded via EOG) when a compound binds to a receptor molecule and initiates a transduction mechanism.

EOG cross-adaptation studies have been used to demonstrate separate olfactory mechanisms for amino acids in the catfish, *Ictalurus punctatus* (Caprio and Byrd, 1984), bile acids in sea lamprey (Li and Sorensen, 1997) and steroidal and prostaglandin hormonal pheromones in the goldfish (Sorensen *et al*., 1995b).

Female hormonal pheromones in other fish species:

Most of the information available on female sex pheromones is based on fish that use a scramble competition mating system in which small groups of non-territorial males compete for access to a single female (Emlin and Oring, 1977); parental care of

fertilized eggs is non-existent. Of the fish species that use a scramble competition mating system, the goldfish is the best understood (Stacey *et al*, 1994a), but it appears that pheromones of the common carp (Irvine and Sorensen, 1993; Stacey *et al*, 1994b), and crucian carp, *Carassius carassius* (Bjerselius *et al*., 1995b) are similar to those of the goldfish. In all three species, males respond to pre-ovulatory steroids and post-ovulatory prostaglandins released by females.

The steroid pheromone is released by female goldfish during final oocyte maturation and has three identified components: the goldfish maturation-inducing steroid (MIS) 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), its 20-sulphated conjugate (17,20β-P-20-s) and androstenedione (AD) (Stacey, 1987; Stacey *et al*., 1989; Scott and Sorensen, 1994; Scott and Vermeirssen, 1994; Sorensen and Scott, 1994). Plasma levels of all three steroids increase in response to a preovulatory gonadotropin-II (GtH-II) surge, and female goldfish release these steroids to the water as the surge progresses (Stacey, 1987; Stacey *et al*., 1989; Scott and Sorensen, 1994; Scott and Vermeirssen, 1994; Sorensen and Scott, 1994). Based on studies of rainbow trout, *Oncorhynchus mykiss*, the route of pheromone release is determined by steroid conjugation (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996). The polar 17,20β-P-20-s is likely released in the urine or feces, whereas the unconjugated 17,20β-P and AD are released via the gills, or other non-urinary routes (Stacey and Cardwell, 1995). The plasma ratio of 17,20β-P to AD increases as the GtH-II surge progresses, and falls dramatically at the onset of ovulation (Sorensen and Scott, 1994). Release of these compounds by the female, together with release of 17,20β-P-20-s by an alternate route, should provide a nearby

male with a clear signal of impending ovulation.

EOG recording demonstrates that the goldfish olfactory system detects 17,20β-P, 17,20β-P-20-s and AD (Sorensen *et al.*, 1990), and detection of 17,20β-P and 17,20β-P-20-s causes an increase in plasma levels of GtH-II in males (Dulka *et al.*, 1987). The surge in GtH-II then increases milt (sperm and seminal fluid) production in preparation for female ovulation and egg release (Dulka *et al.*, 1987). When goldfish are exposed to a mixture of AD and 17,20β-P, the physiological effect of 17,20β-P is blocked, indicating that the ratio of 17,20β-P to AD is an important part of the signal (Stacey, 1991; Scott and Sorensen, 1994; Sorensen and Scott, 1994).

The postovulatory pheromone in goldfish is comprised of prostaglandin F2α (PGF2α) and its metabolites. Once eggs have ovulated, plasma levels of the maturational steroid fall abruptly, and levels of prostaglandin F2α (PGF2α) rise (Sorensen *et al.*, 1995a). Prostaglandins are believed to be produced in response to the presence of ovulated oocytes in the reproductive tract (Sorensen *et al.*, 1995a). The elevated concentration of PGF2α in the blood stimulates female sexual activity (Stacey, 1987). PGF2α and metabolites are released to the external environment (Sorensen *et al.*, 1995a), and are detected by the male olfactory system (Sorensen *et al.*, 1988) which then triggers male courtship behaviour (Stacey, 1981; Sorensen *et al.*, 1986, 1989). Prostaglandin exposure also stimulates a rapid increase in plasma levels of GtH-II and milt volume in male goldfish (Kyle *et al*, 1985; Sorensen *et al.*, 1989; Zheng and Stacey, 1996).

Prostaglandins and PG metabolites also apparently function as post-ovulatory pheromones in other teleosts, such as the loach, *Misgurnus anguillicaudatus*, a cyprinid

in which male sexual behaviour is stimulated by exposure to females injected with PG (Kitamura *et al.*, 1994a). The loach is reported to release high levels of prostaglandins and PG metabolites at ovulation (Ogata *et al.*, 1994) and the olfactory system exhibits sensitivity to PGs (Kitamura *et al.*, 1994b). EOG studies on Atlantic salmon (*Salmo salar*), a species that exhibits a mating strategy different than goldfish, in that males are territorial and aggressive during spawning, demonstrate that the olfactory system of males is sensitive to PGs. Male parr also exhibit increases in plasma GtH-II, androgens and 17,20β-P after exposure to PGs that are thought to be released by ovulated females in urine (Moore and Waring, 1995; Waring and Moore, 1995).

Prostaglandins may be female-specific pheromones, as sexually mature males of several species exhibit greater sensitivity to PGs than do females: eg. goldfish (Sorensen and Goetz, 1993), common carp (Irvine and Sorensen, 1995) crucian carp (Bjerselius and Olsen, 1993), Mexican blind cavefish, *Astyanax mexicanus* (Cardwell and Stacey, 1995b), and tinfoil barbs (*Puntius schwanenfeldi* and *Puntius gonionotus*; Cardwell *et al.*, 1995). In *Puntius*, olfactory sensitivity to PGs has been demonstrated to be modulated by androgens; juvenile *Puntius* implanted with methyl testosterone (MT) showed enhanced olfactory sensitivity (determined via EOG) to PGs in comparison to non-implanted juveniles (Cardwell *et al.*, 1995).

EOG surveys of a diversity of fish species show that many species of fish from the Orders Cypriniformes, Characiformes and Siluriformes are sensitive to prostaglandins, indicating that the pheromonal function of PGs in these groups is likely widespread (Stacey and Cardwell, 1995). Interestingly, no species in the Order Perciformes, which

includes the round goby, has been found to detect PGs (Stacey and Cardwell, 1995).

Male hormonal pheromones in other fish species:

In addition to the black goby, other fish species are reported to use steroid glucuronides as male hormonal pheromones. In male African catfish (*Clarias gariepinus*), specialized interstitial cells of the seminal vesicle and testis synthesize steroid glucuronides which attract ovulated females (van den Hurk and Resink, 1992). The identity of the putative steroidal pheromone has been proposed to consist of free and conjugated 5β-reduced androgens and a C21 conjugate (5β-pregnan-3α,17α-diol-20-one-3α-glucuronide (3α,17α-5βP-3g) (Resink *et al.*, 1989a). This mixture of steroids was extracted from incubation medium containing seminal vesicles fragments of the male African catfish, and ovulated females were significantly attracted to a mixture of synthetic versions of the same steroids (Resink *et al*, 1989a). Additionally, EOG recording demonstrated that each steroid in the mixture was detected by the olfactory system of the African catfish. Two steroids isolated from the seminal vesicles, 3α,17α-5βP-3g and 5β-androstan-3α,11β-diol-17-one-3-glucuronide (11β-Etio-g), are potent olfactory stimulants with detection thresholds as low as 10^{-11} M (Resink *et al*, 1989b). Whether the African catfish only responds to mixtures of detected steroids is not known, as individual steroids were not tested as attractants (Resink *et al.*, 1989).

Steroid glucuronides are also reported to induce ovulation in female zebrafish, *Brachydanio rerio* (van den Hurk *et al.*, 1987; van den Hurk and Resink, 1992). Male zebrafish do not have accessory glands. The steroid glucuronides that act as pheromones

are believed to be produced in the testis, as glucuronide fractions from testicular homogenates will induce ovulation in females. The steroid conjugates synthesized by the testis were identified as primarily 5α-reduced steroid glucuronides, as well as a few C21 conjugates (one being 17,20β-P-g) (van Den Hurk *et al.*, 1987). However, EOG recordings demonstrate that the olfactory system of zebrafish does not detect the identified 5α-reduced steroid glucuronides and 17,20β-P-g (Stacey and Cardwell, 1995).

Steroid conjugates may be released in the urine, as male urine attracts ovulated female *G. jozo* (Colombo *et al,* 1982). Urine has been shown to be a major route of excretion of conjugated steroids in other species, such as the rainbow trout (Scott and Liley, 1994), plaice, *Pleuronectes platessa*, Dover sole, *Solea solea* (Scott and Canario, 1992), herring, *Clupea harengus* (Scott *et al.*, 1991a), and Atlantic salmon (Moore and Scott, 1991). Male urine is proposed to be the source of a pheromone in the yellowfin Baikal sculpin *(Cottocomephorus grewingki)*, another nest-guarding, paternal care, territorial species (Dmitrieva and Ostroumov, 1986; Dmitrieva *et al.*, 1988; Katsel *et al.*, 1992). In this species, the volume of the male bladder increases approximately five-fold during the spawning season. The bladder may be specialized towards signal production and release of large quantities of steroids like 11β-hydroxytestosterone (KT) and testosterone (T). KT was proposed to promote ovulation in the sculpin, and then trigger female spawning when released with T at nesting sites (Dmitrieva and Ostroumov, 1986; Dmitrieva *et al.*, 1988; Katsel *et al.*, 1992). However, studies on rainbow trout show that unconjugated T and KT are not released in the urine but via the gills or other non-urinary routes (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996); thus, it is uncertain

whether T and KT are released in the urine in the yellowfin sculpin.

In addition to urine, milt may also be a pheromone source. Milt triggers spawning behaviour in ovulated and spermiated Pacific herring, *Clupea harengus pallasi* (Stacey and Hourston, 1982). Hormonal pheromones may also be released with the milt, as high levels of free and conjugated steroids (eg. 17,20β-P and cortisol) were isolated from seminal fluid of Pacific herring and North sea plaice (Scott *et al.*, 1991b), and rainbow trout (Loir, 1990).

Objectives of this study:

The objectives of this research were threefold: 1) to identify, using EOG recording, hormone and hormone metabolites detected by *Neogobius*; 2) to determine, using EOG and behavioural cross-adaptation techniques, whether detected compounds act on more than one olfactory receptor mechanism; and 3) to identify reproductive responses induced by the detected compounds. Although the third objective was not met, results of the first two objectives indicate that *Neogobius* detects a variety of free and conjugated steroids, and can discriminate a number of these at both the sensory and behavioural level. Furthermore, the sexual dimorphism exhibited in behavioural responses to detected steroids indicates that the potential pheromones may function in reproductive signalling. The findings suggest a complex hormonal pheromone system in *Neogobius* and make a strong foundation for future studies.

2. FISH

N. melanostomus were collected between May and August, 1994-96 by angling and bottom trawl from the St. Clair and Detroit Rivers (Windsor, Ontario). The fish were shipped by air to Edmonton, where they were maintained under constant photoperiod (16L:8D) in 70L aquaria with flowing dechlorinated tapwater at controlled temperatures roughly equivalent to seasonal field temperature (9°C-18°C from winter to summer). Aquaria contained gravel, an airstone, artificial weed cover, and sheltered spaces. A variety of live, frozen and flake food was provided *ad libitum*

Fish were sexed by examining the shape of their urogenital papilla. The papilla of female fish has a broad, truncated tip with a large pore, whereas that of males is long and slender with a minute opening (Tavolga, 1954). Dissection of fish confirmed this differentiation. Males also had mesorchial glands, specialized Leydig cell-rich testicular structures common in male gobiids (Miller, 1986). Experiments performed on fish followed Canadian Council of Animal Care approved procedures (C.C.A.C, 1993).

3. OLFACTORY RESPONSE TO PUTATIVE STEROIDAL PHEROMONES

3.1. METHODS

3.1.1. Electro-olfactogram (EOG) procedure

Sexually mature male and female *Neogobius* were tested for olfactory sensitivity to a variety of odours using electro-olfactogram (EOG) recording procedures similar to those described by Cardwell *et al.* (1995). Fish were initially anesthetized by orally perfusing the gills with dechlorinated tapwater containing 0.05% 2-phenoxyethanol (2-PE: Sigma. St. Louis MO) via a mouth tube. Fish were then wrapped in wet paper tissue, secured to a stand, placed in an electrically grounded water bath, and fitted with a polyethylene mouth tube which delivered aerated, dechlorinated water (containing 0.05% 2-PE) throughout the entire procedure. The temperature of the water bath and the anesthetic water approximated that of field ambient temperature (9°C-18°C from winter to summer).

A glass capillary tube (70-120 µm tip diameter) filled with gelatin (8% in 0.6% NaCl) bridged Ag/AgCl electrodes filled with 3M KCl to the olfactory tissue. The tip of the reference electrode was placed in the water bath, while the recording electrode was placed just inside the incurrent pore of the naris. An odour delivery tube was positioned next to the open excurrent pore of the naris allowing water to flow in the excurrent and out the incurrent. It was necessary to use a reversed water flow through the naris because recordings could be obtained only when the electrode was placed in the incurrent pore.

Because some fish were subjected to more than one EOG recording session, the tissue overlaying the olfactory organ was not removed to avoid damaging the olfactory epithelium.

Throughout a recording procedure, the naris was continuously perfused with dechlorinated tapwater (background) until a computer-controlled solenoid switched the background solution to a test solution for two seconds. The amplified signals (Grass P-18 DC amplifier) from the electrodes were digitized (National Instruments Lab-PC A/D converter), and the absolute response was recorded for the ten seconds following initial exposure to the test odour. Fish were anesthetized for a maximum of three hours and, after recording was completed, gills were perfused with tapwater without anesthetic until the fish began ventilating and could be returned to their aquaria. Fish were allowed to recover for at least one week before being re-tested. Individual fish were tested a maximum of three times, and each recording was for a different cross-adaptation experiment. There was no evidence that the EOG was affected by prior recordings, as response magnitudes to odours remained approximately equivalent between tests.

EOG recording was not begun until the fish had been mounted in the water bath for 30-45 minutes. At this point, olfactory sensitivity to the internal standard (10^{-5} M L-alanine) and to the background test solution of dechlorinated tapwater was recorded. Internal standards, such as 10^{-5} M L-alanine, that are detected via different olfactory mechanisms than hormonal pheromones, and are detected by all fish (Hara, 1992), are necessary in EOG screenings of many compounds that are likely not detected. A response to the internal standard ensures that the EOG preparation is functioning

normally, and provides a means to monitor the stability of a recording over time. Also, responses to an internal standard can be used to normalize responses obtained from separate individuals, as each response to a test compound can be calculated as a percentage of the internal standard. In these EOG recordings, a response to 10^{-5} M L-alanine that was greater than 3 mV indicated that the electrodes and odour delivery tube had been positioned correctly. If the response was less than 3 mV, the electrodes and odour tube were repositioned until a 3 mV response was obtained, or the recording was terminated.

During the course of a recording, the sensitivity to 10^{-5} M L-alanine was monitored frequently to assess the quality of the recording. As well, the naris was exposed occasionally to 2-sec pulses of the same dechlorinated tapwater (background water) that normally irrigated the naris. The response to background water (usually < 0.1 mV) was subtracted from the response magnitudes of all subsequently tested odours, and this background water correction was adjusted the next time a new background water was tested. Data (base to peak voltage differences) were presented as either an absolute response in mV, or as a percentage of a response to a 10^{-5} M L-alanine standard. Adaptation was minimized by allowing 1-2 minutes between exposure to test solutions.

3.1.2. Tested Odours

The olfactory epithelium of *Neogobius* was exposed to 114 steroids and eight prostaglandins (Appendix A) at 10^{-8} M to determine which compounds were olfactory stimulants. Steroids and prostaglandins were obtained from various sources. Steroids

and steroid conjugates were purchased from Sigma, St. Louis MO and Steraloids, Wilton NH, and as gifts from Dr. A. P. Scott (Lowestoft), and Dr. J. G. D. Lambert (Utrecht). Prostaglandins were purchased from Cayman Chemical Company, Ann Arbor, MI. Many of the tested steroids and prostaglandins listed in Appendix A are known or putative pheromones in other species of teleosts, or are related to these olfactory stimulants (Stacey and Cardwell, 1995).

The L-alanine standard (Sigma, St. Louis MO), was prepared as a 10^{-2} M stock solution in double-distilled de-ionized water and stored in glass scintillation vials at 4°C. The stock was diluted to 10^{-5} M in background water when used in the EOG recording. Olfactory response to 10^{-6} M L-alanine was also recorded, as this concentration was used in behaviour tests.

All other test odours were first prepared as stock solutions (10^{-3} M) in 99% ethanol (free and sulphated steroids, prostaglandins) or 50:50 ethanol:water (glucuronated steroids). All stock solutions were stored at -20°C. 10^{-5} M working solutions, in duplicate, were prepared by diluting stock solutions into double-distilled, de-ionized water, and stored in glass scintillation vials at 4°C. Test solutions were prepared immediately before recording by diluting working solutions in 100 ml of background water (Cardwell *et al.*, 1995).

Five gobies (2 males, 3 females) were exposed to 10^{-8} M test solutions of all the compounds listed in Appendix A. Compounds that did not elicit a response from any fish were considered undetectable, and were not tested again. A compound was considered detectable if its EOG response exceeded the voltage change induced by the background

solution, and if it induced a response in more than one fish. Additionally, to ensure a response to a compound was not due to a contamination of the test solution, each detected steroid was tested with a fresh test solution. As a control, the amount of ethanol solvent in 10^{-8} M test solutions (10 µl) was added to 100 ml of background solution and tested for responsiveness.

3.1.3. EOG concentration response studies

To compare olfactory potency of detected steroids, concentration response relationships were determined for six steroids. Steroids chosen for the concentration response study were steroids that elicited strong responses at 10^{-8} M (greater than 20% of the response to the 10^{-5} M L-alanine standard), and steroids that may operate via separate receptor mechanisms (see below). Although the response to Etio-g (10^{-8} M) was less than 20% of the response to alanine standard, it was studied because it has been proposed to have pheromonal function in *G. jozo* (Colombo *et al*, 1982).

At this stage, criteria for determining compounds that may act on different receptors were arbitrarily based on studies in goldfish showing that 19 and 21 carbon steroids act via different receptor mechanisms (Sorensen *et al*., 1990), and that some free and conjugated steroids also act via separate mechanisms (Sorensen *et al*., 1995b). I therefore assumed that, in *Neogobius*, 18 carbon steroids might act via a different receptor than 19 carbon steroids, and that a free steroid might bind to a different receptor than a glucuronated or sulphated conjugate. The steroids chosen for concentration response studies were: estrone (E1), estradiol-glucuronide (E2-3g), etiocholanolone (Etio), Etio-g,

androsterone-sulphate (Andr-s), dehydroepiandrosterone-sulphate (DHEA-s) and dehydroepiandrosterone-glucuronide (DHEA-g).

Concentration response tests began at 10^{-12} M and increased by log molar increments to 10^{-8} M, with 10 minute intervals between each exposure. Each testing solution was brought to the appropriate concentration by diluting aliquots of stored 10^{-5} M solutions in background water.

Concentration response tests for each selected steroid were performed on six fish (4 females, 2 males). Response magnitudes were recorded as mV and analyzed using ANOVA and Tukey multiple comparison test (Systat, 1994).

3.1.4. EOG cross-adaptation

EOG cross-adaptation studies were conducted to determine the quantity and nature of olfactory receptor mechanisms responsive to putative pheromones. These studies compare the EOG response of a test compound before and during adaptation to another compound. If the EOG response is unaffected by adaptation, it is assumed that the test and adapting compounds operate through separate receptor mechanisms (Caprio and Byrd, 1984; Sorensen *et al*, 1995b; Li and Sorensen, 1997).

The design of the standard cross-adaptation procedure makes the assumption that a brief exposure to an odour will not influence EOG response to that odour when presented a short time later. However, interpretation of a cross-adaptation experiment will be confounded if a tested odour elicits a smaller response on the second presentation, even in the absence of a tonically-delivered adapting odour. To evaluate the extent of this

phenomenon in *Neogobius*, six fish (4 females, 2 males) were exposed sequentially to 2 second 10^{-8} M pulses of all steroids used for the cross-adaptation studies, and then re-tested with the same steroids 30 minutes later. Response to the alanine standard was monitored throughout the experiment and it was found to remain at a constant magnitude. All changes in response magnitude between first exposure and second exposure were compared using a Wilcoxon matched-pairs test for non-parametric data (Systat, 1994). Decreases in magnitude of the response to the second exposure were calculated as percent changes using the following formula :

((Response (1st) - Response (2nd))/ Response (1st)) x 100

The cross-adaptation procedure was similar to that described by Sorensen *et al.* (1995). Initially, the naris was sequentially exposed to 10^{-8} M solutions of all of the detected compounds (Table 3.1) to establish pre-adaptation responses. Then, while the naris was exposed to a 10^{-7} M solution of the adapting compound, the 10^{-8} M test steroids were administered again, and in the same sequence. Test steroids were used at 10^{-8} M because many of them did not induce an EOG response at lower concentrations.

Five adapting solutions were chosen: E1, E2-3g, DHEA-g, Etio and DHEA-s. While it is possible to use more than five adapting solutions, time constraints forced an arbitrary selection of compounds that I assumed would operate via separate receptor mechanisms. Preliminary cross-adaptation experiments also indicated which compounds might operate via separate receptors. I also chose compounds that were detected at lower concentrations, determined from concentration-response studies. Cross-adaptation experiments for each adapting steroid were conducted on 6 fish (4 females, 2 males).

To monitor the quality of the recording during the cross-adaptation procedure, the naris was exposed to 10^{-5} M L-alanine at the beginning and the end of each cross-adaptation test, as well as after every fourth steroid exposure. If the response to alanine (10^{-5} M) response dropped below 3 mV, the recording electrode and odour delivery tube were repositioned until a strong alanine response was regained. In approximately 40% of the cross-adaptation experiments, the response to alanine (10^{-5} M) could not be recovered, and the test was terminated.

To avoid adaptation during the pre-adaptation testing, the compounds known to have the smallest response magnitudes were tested first. Control cross-adaptations, sequential 2-sec odour presentations given 30 min apart, showed that some tested odours elicit a smaller response on the second presentation in the absence of an adapting solution, and responses to steroids that induce small response magnitudes might be masked by a previous exposure to a compound that acts via the same receptor. If a steroid failed to elicit a second response in the control cross-adaptation, it was eliminated from all cross-adaptation studies (see section 3.2.3). Also, some of the steroids that induced small responses at 10^{-8} M, failed to elicit a response in the pretest; in these situations, the compound was not tested again during the actual cross-adaptation phase. As well, compounds of similar structure were not tested in sequence to reduce the possibility of adaptation by exposure to multiple compounds that might operate via the same receptor mechanism. The same sequence of exposure to detected compounds was used for all pretest and cross-adaptation exposures in each cross-adaptation experiment, as well as in the control cross-adaptation tests.

The adapting solution was prepared as a 2 litre solution of the adapting steroid (10^{-7} M) dissolved in background tapwater. The same adapting solution was used to adapt the naris and to prepare dilutions of the test steroids. The naris was exposed to the adapting solution until the induced response declined to a stable plateau (usually after 3 minutes), after which responsiveness to test steroids dissolved in adapting solution was determined. Absolute response magnitudes (mV) were used to calculate percent change in response between pretest and cross-adaptation response using the following formula:

((Response (pre) - Response (crad))/ Response (pre)) x 100

where crad = cross-adaptation response, pre = pretest response.

To determine if adaptation significantly affected response to a test steroid, the percent reduction in response during adaptation was compared to the percent reduction observed in control treatments where the same compound was applied twice, but without adaptation. Differences between control and adapting reductions were analyzed by Mann Whitney U test.

3.2. RESULTS

3.2.1. Response to test odours

Neogobius consistently showed EOG responsiveness to L-alanine (10^{-5} M: 8.0±5.1 mV (mean±SD), range (2.9-21.9 mV), N=25; 10^{-6} M: 3.1±1.7 mV (mean±SD), range (1.0-4.8 mV), N=8), with no difference between males and females in response to 10^{-5} M L-alanine (7.4±3.8 (mean±SD) vs 8.3±5.7 (mean±SD); P>0.10; males (N=8), females (N=17)). Responses to 10^{-8} M E2-3g were significantly correlated to 10^{-5} M L-alanine responses (Pearson r=0.655, P<0.01, N=25), justifying the use of 10^{-5} M L-alanine as an internal standard. Tests using background water to determine if there were any mechanical artifacts did not elicit a response from the olfactory epithelium (0.00-0.10 mV in all cases) (Fig. 3.1). Of the 114 steroids and 8 prostaglandins tested (Appendix A), 19 steroids induced responses significantly greater than those induced by background water; none of the prostaglandins induced an EOG response (Table 3.1). All responses to 10^{-8} M steroids were less than 60% of the alanine response (Fig. 3.1, Table 3.1). Because for each steroid, EOG responses of males and females were similar in magnitude (Fig. 3.2), EOG results from both sexes were pooled.

At a concentration of 10^{-8} M (the highest concentration tested), a diverse array of free and conjugated C18, C19 and C21 steroids induced an EOG response. The C18 steroids, such as estrone (E1), elicited the largest EOG response (Table 3.1, Fig. 3.2). The detected C19 steroids form a diverse group: 4-androstene- (AD), 5-androstene (DHEA-g, and DHEA-s), 5β-androstan (Andr-s, and Epiandr-g), and 5α-androstan (Etio, Etio-g, 11β-Etio-g, and Etio-3,17-dione). All the detected C21 steroids were variations

and conjugates of the 5β-pregnan stem. The amount of ethanol solvent in a 10^{-8} M steroid solution did not elicit an EOG response.

3.2.2. EOG concentration response results

For some fish, threshold concentration for E1, E2-3g, Etio and Etio-g was 10^{-11} M. However, group responses to steroid concentrations were not significantly different ($P<0.05$) than background until 10^{-9} M for E1, E2-3g, and Etio, and 10^{-8} M for Etio-g (Fig 3.3). Andr-s, and DHEA-g reached threshold at 10^{-9} M and were significantly different than background at 10^{-8} M (Fig. 3.3e, f). Threshold concentration for DHEA-s was 10^{-8} M (Fig. 3.3g). Once the detection threshold concentration for each steroid was reached, all concentration response profiles increased in magnitude with concentration (Fig. 3.4).

3.2.3. EOG cross-adaptation results

When the steroids used in the cross-adaptation studies were delivered as 2 second 10^{-8} M pulses 30 min apart, DHEA-g elicited significantly smaller EOG responses ($P<0.05$) on the second presentation (Fig. 3.5). Although few replicates were conducted, several 5β-pregnan compounds (5βP; 3α,20β-5βP; 3β,20β-5βP) did not elicit any response on the second presentation, and therefore were omitted from cross-adaptation studies. Although EOG responses to C18 compounds were not significantly reduced on the second presentation, there was a consistent trend for reduced response magnitude within this group of compounds (Fig. 3.5). Thus, to determine if the reduction in cross-

adaptation response was due to the interaction of the adapting solution, and not due to the reduced responsiveness on second presentation of an individual odour, the differences observed between the pretest response and the cross-adaptation response were compared to the differences observed in the control test. For example, to determine if Etio significantly adapted olfactory responsiveness to E1, the percent reduction in magnitude observed in all the control cross-adaptation responses to E1 was calculated using the following formula:

((E1 response 1st exposure - E1 response 2nd exposure)/ E1 response 1st) x 100

The percent reduction in magnitude observed in all responses to E1 while being adapted by Etio was calculated as follows:

((Pretest response to E1-Adapted response to E1)/Pretest response to E1) x 100

The percent reductions in magnitude observed in the E1 control cross-adaptation test were then compared to the percent reductions observed in E1 when Etio was the adapting solution using a Mann-Whitney U test. If the differences were significant, then the adapting solution may have interacted with the steroid, indicating that the two compounds were acting via the same receptor mechanism. Only significant differences between control tests and cross-adaptation tests will be discussed.

Results of all cross-adaptation tests suggest that *Neogobius* possesses four different receptor mechanisms for steroids (Figs. 3.7 and 3.8). Cross-adaptation tests indicate the C18 compounds are detected by two receptor mechanisms that are most sensitive to E1 and E2-3g respectively. Adaptation to 10^{-7} M E1 (Figs 3.6 and 3.7a) reduced or eliminated EOG responses to E1, E2, and E2α but did not significantly reduce

the response to E2-3g, or to any other tested steroid (Fig. 3.7a). Further evidence that E2-3g acts through a receptor mechanism different from that detecting E1 is the finding that, during adaptation to E2-3g, EOG responses to E1, E2, and E2α were not significantly reduced (Fig 3.7b). The E2-3g receptor also appears to interact with DHEA-g and Epiandr-g because, during adaptation to E2-3g or DHEA-g, EOG responses to these compounds were reduced. (Fig 3.7b, c). Adaptation to DHEA-g significantly reduced EOG responses to both E2-3g and Epiandr-g.

Cross-adaptation results also indicate an additional two separate receptor mechanisms that are most responsive to Etio and DHEA-s (Fig 3.8). For example, during adaptation to 10^{-7} M Etio, EOG response to a number of androstan compounds (AD, Andr-s, Etio-3,17-dione, Etio, Etio-g, and 11β-Etio-g) and C21 steroids (3α,17α-5βP, 3α, 17α,20β-5βP, and 3α,17α-5βP-3g) were significantly reduced ($P<0.05$) (Fig. 3.8a). Epiandr-g was not tested in this cross-adaptation study, because it was not discovered that the goby detects this compound until after this cross adaptation study was completed. The receptor mechanism for Etio appears to be distinct from the receptor mechanism of DHEA-s, although adaptation to DHEA-s (Fig. 3.8b) significantly reduced EOG responses to some of the same compounds (e.g. Andr-s and 3,17-5βP-3g) that were reduced during adaptation to Etio (Fig 3.8a). DHEA-s (10^{-7} M) decreased the cross-adaptation response to 10^{-8} M DHEA-s, but did not alter the cross-adaptation response to any of the other steroids except Andr-s and 3,17-5βP-3g; the latter two steroids may operate via two receptor mechanisms, the Etio receptor and the DHEA-s receptor.

The results of the cross adaptation study are summarized in Tables 3.2 and 3.3.

The detected steroids appear to act through at least four distinct receptor classes, each named for the steroid that induced the largest EOG response when tested at 10^{-8} M (Table 3.1). Three compounds that appear to interact with more than one receptor class are listed separately (Table 3.3). More work is required to determine which receptor class is stimulated by Epiandr-g.

Table 3.1. Detected steroids, grouped according to chemical structure with EOG responses at 10^{-8} M (% Alan), and their functions in other teleosts; s.d. (*N*)= number of determinations.

Chemical Structure	Compound	Abbreviation	Resp. at $^{-8}$ M (mean %Alan)	Reported function
C18 estratrienes	1,3,5[10]-Estratriene-3-ol-17-one **estrone**	E1	56.0±29.1 (*24*)	unknown
	1,3,5[10]-Estratriene-3,17β-diol **β-estradiol**	E2	54.0±28.7 (*23*)	unknown
	1,3,5[10]-Estratriene-3,17α-diol **17α-estradiol**	E2α	27.2±20.8 (*24*)	unknown
	1.3.5[10]-Estratriene-3,17β-diol-3-glucuronide **17β-estradiol-3-glucuronide**	E2-3g	48.7±35.9 (*25*)	attracts male zebra-fish (Van den Hurk, Lambert)
C19 4-androsten	4-androsten-3,17-dione **androstenedione**	AD	9.7±8.1 (*20*)	inhibits GtH in male goldfish (Stacey *et al*)
C19 5α-androstan	5α-androstan-3β-ol-17-one-3β-glucuronide **epiandrosterone-glucuronide**	Epiandr-g	17.6±1.8 (*4*)	unknown
	5α-androstan-3α-ol-17-one-3-SO4 **androsterone-sulphate**	Andr-s	46.8±26.5 (*25*)	unknown
C19 5β-androstan	5β-androstan-3α-ol-17-one **etiocholanolone**	Etio	23.5±23.5 (*20*)	unknown
	5β-androstan-3α-ol-17-one-3-glucuronide **etiocholanolone-glucuronide**	Etio-g	15.3±19.8 (*20*)	attracts female *G. Jozo* and stimulates oviposition (Colombo *et al)*
	5β-androstan-3,17-dione **etiocholane-3, 17-dione**	Etio- 3,17-dione	20.1±12.0 (*24*)	unknown
	5β-androstan-3α,11β-diol-17-one-3-glucuronide **11β-Hydroxyetiocholanolone-gluc**	11β-Etio-g	14.2±6.7 (*10*)	unknown
C19 5-androsten	5-androsten-3β-ol-17-one-3-glucuronide **dehydroepiandrosterone-glucuronide**	DHEA-g	17.4±15.1 (*23*)	unknown
	5-androsten-3β-ol-17-one-3-SO4 **dehydroepiandrosterone-sulphate**	DHEA-s	20.9±19.8 (*20*)	unknown

Chemical Structure	Compound	Abbreviation	Resp. at 10^{-8} M (mean %Alan)	Reported function
C21 5β-pregnan	5β-pregnan-3,20-dione	5βP	12.7±13.5 (*20*)	unknown
	5β-pregnan-3β,20β-diol	3β,20β-5βP	16.5±15.9 (*15*)	unknown
	5β-pregnan-3α,20β-diol	3α,20β-5βP	6.6±10.3 (*14*)	unknown
	5β-pregnan-3α,17-diol-20-one	3α,17α-5βP	13.8±16.3 (*19*)	unknown
	5β-pregnan-3α,17α,20β-triol	3α,17α,20β-5βP	9.0±8.4 (*20*)	unknown
	5β-pregnan-3α,17α-diol-20-one-3α-glucuronide	3α,17α-5βP-3-g	31.0±19.2 (*20*)	unknown

Table 3.2. Steroids detected by olfactory receptor classes indicated by EOG cross-adaptation studies.

Class	C	Compound	Abbreviation
1	C18	1,3,5[10]-Estratriene-3-ol-17-one **estrone**	E1
		1,3,5[10]-Estratriene-3,17β-diol **β-estradiol**	E2
		1,3,5[10]-Estratriene-3,17α-diol **17α-estradiol**	E2α
2	C18	1.3.5[10]-Estratriene-3,17β-diol-3-glucuronide **17β-estradiol-3-glucuronide**	E2-3g
	C19	5-androsten-3β-ol-17-one-3-glucuronide **dehydroepiandrosterone-glucuronide**	DHEA-g

Class	C	Compound	Abbreviation
3	C19	5-androsten-3β-ol-17-one-3-SO4 **dehydroepiandrosterone-sulphate**	DHEA-s
4	C19	5β-androstan-3α-ol-17-one **etiocholanolone**	Etio
		5β-androstan-3α-ol-17-one-3-glucuronide **etiocholanolone-glucuronide**	Etio-g
	C19	5β-androstan-3,17-dione **etiocholane-3,17-dione**	Etio-3,17-dione
		5β-androstan-3α,11β-diol-17-one-3-glucuronide **11β-Hydroxyetiocholanolone-gluc**	11β-Etio-g

Class	C	Compound	Abbreviation
4	C19	4-androsten-3,17-dione **androstenedione**	AD
	C21	5β-pregnan-3,20-dione	5βP
		5β-pregnan-3β,20β-diol	3β,20β-5βP
		5β-pregnan-3α,20β-diol	3α,20β-5βP
		5β-pregnan-3α,17-diol-20-one	3α,17α-5βP
		5β-pregnan-3α,17α,20β-triol	3α,17α, 20β-5βP

Table 3.3. Steroids detected by more than one olfactory receptor classes indicated by EOG cross-adaptation studies.

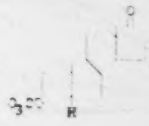
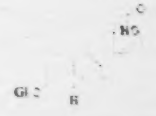
Class	C	Compound	Abbrev.	
3 and 4	C19	5α-androstan-3α-ol-17-one-3-SO4 **androsterone-sulphate**	Andr-s	
	C21	5β-pregnan-3α,17α-diol-20-one-3α-glucuronide	3α,17α-5βP-3g	
unknown possibly 2 and 4	C19	5α-androstan-3α-ol-17-one-3β-glucuronide **epiandrosterone-glucuronide**	Epiandr-g	

Figure 3.1. EOG responses to 10^{-5} M L-alanine (standard), 10^{-8} M estrone and background. Two second odour pulse (real time) indicated by bar.

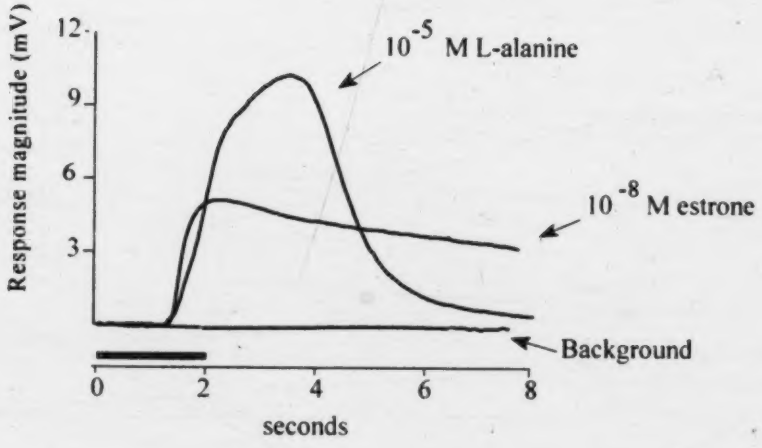

Response magnitude (mV)
12
9
6
3
10^{-5} M L-alanine
10^{-8} M estrone
Background
0
2
4
6
8
seconds

Figure 3.2. Comparison of male and female EOG response magnitudes (mean +s.d.) to detected steroids. Sample size indicated above bars. See Table 3.1 for full names of steroids.

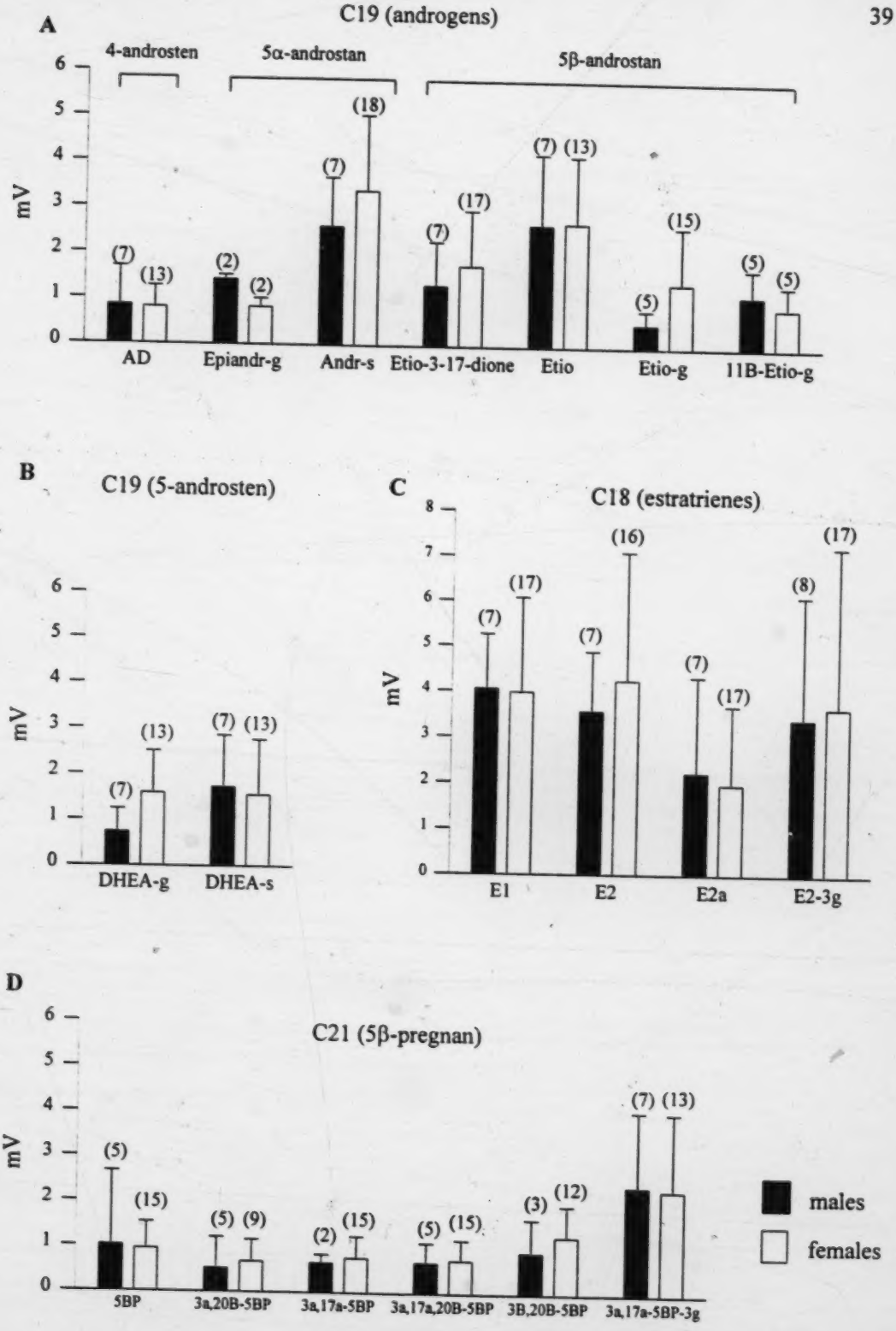
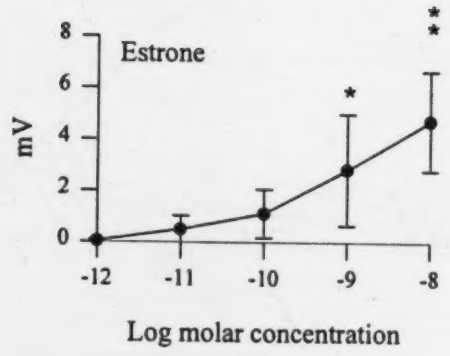
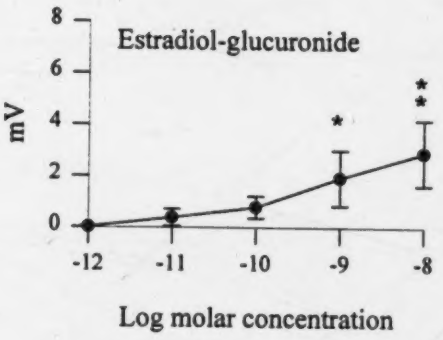
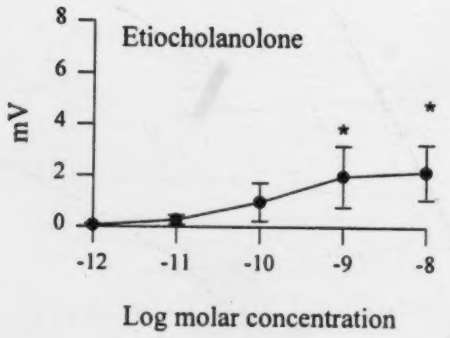
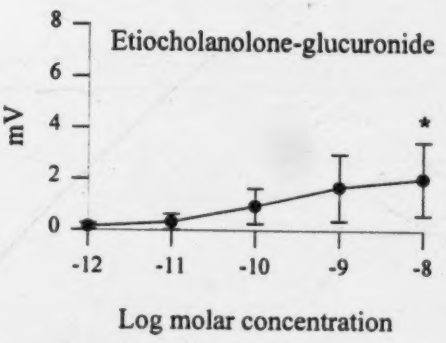

A
C19 (androgens)
4-androsten
5α-androstan
5β-androstan
mV
AD
Epiandr-g
Andr-s
Etio-3-17-dione
Etio
Etio-g
11B-Etio-g
B
C19 (5-androsten)
DHEA-g
DHEA-s
C
C18 (estratrienes)
E1
E2
E2a
E2-3g
D
C21 (5β-pregnan)
5BP
3a,20B-5BP
3a,17a-5BP
3a,17a,20B-5BP
3B,20B-5BP
3a,17a-5BP-3g
males
females

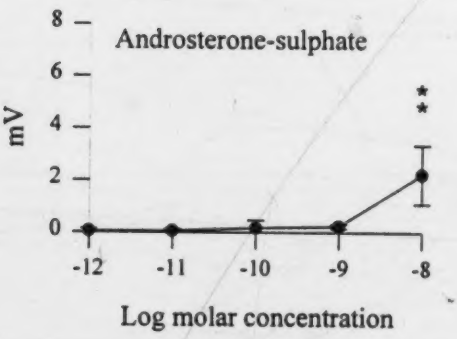
Figure 3.3. Concentration response profiles (mean + s.d.) for selected steroids. EOG responses expressed in mV. N=6 for each steroid. Significant difference compared to background water indicated by * (P<0.05) or ** (P<0.01).

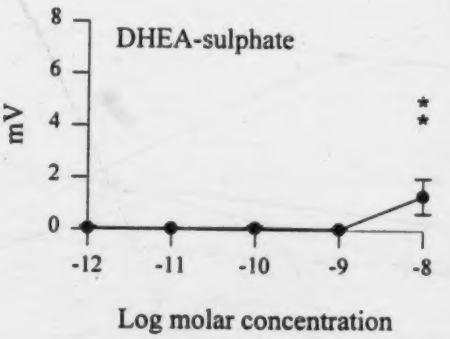

Estrone
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration


Estradiol-glucuronide
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration


Etiocholanolone
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration


Etiocholanolone-glucuronide
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration


Androsterone-sulphate
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration


DHEA-sulphate
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration

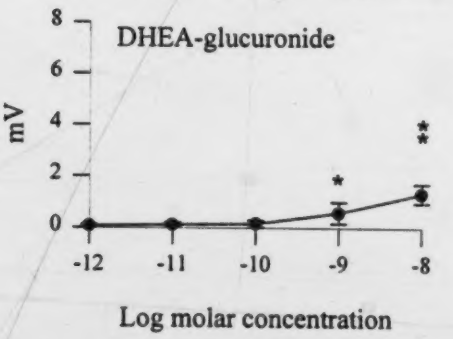

DHEA-glucuronide
mV
8
6
4
2
0
-12
-11
-10
-9
-8
Log molar concentration

Figure 3.4. Typical EOG concentration response profile for estrone. Two second odour pulse (real time) indicated by bar.

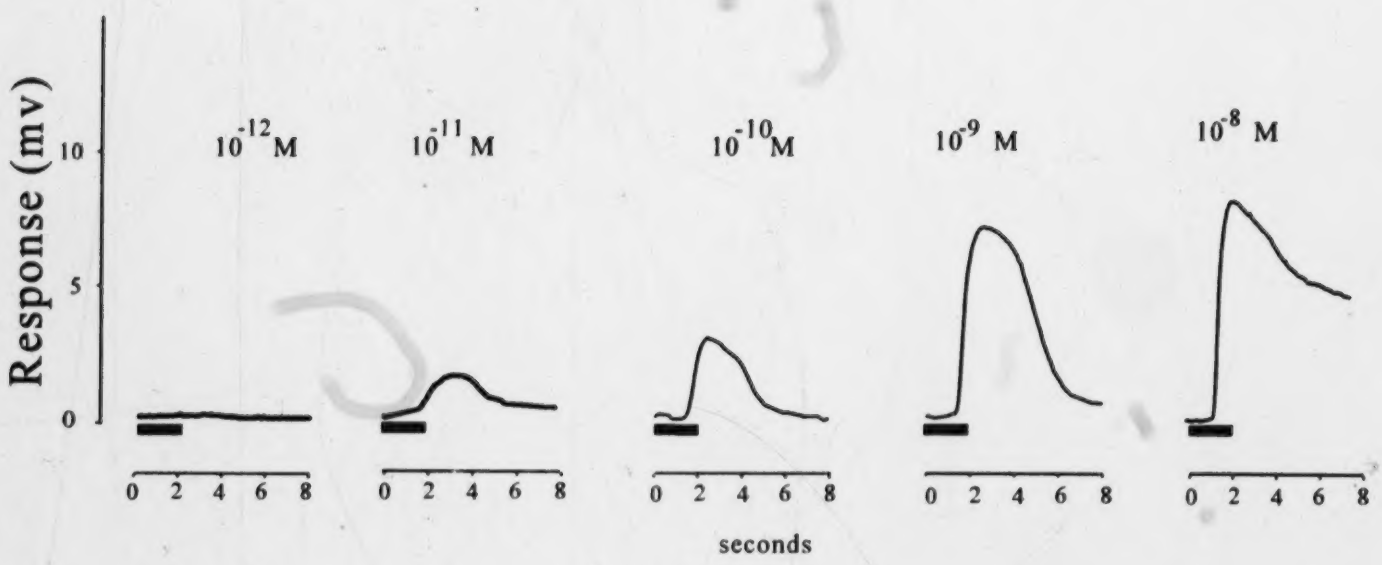

Response (mv)
10
5
0
10^{-12} M
10^{-11} M
10^{-10} M
10^{-9} M
10^{-8} M
0 2 4 6 8
seconds

Figure 3.5. EOG response (mean + s.d.) to 2-sec 10^{-8} M steroid pulses delivered 30 min apart. Sample size indicated beside bars. See Table 3.1 for full names of steroids. *= magnitude of response to second pulse significantly reduced ($P<0.05$).

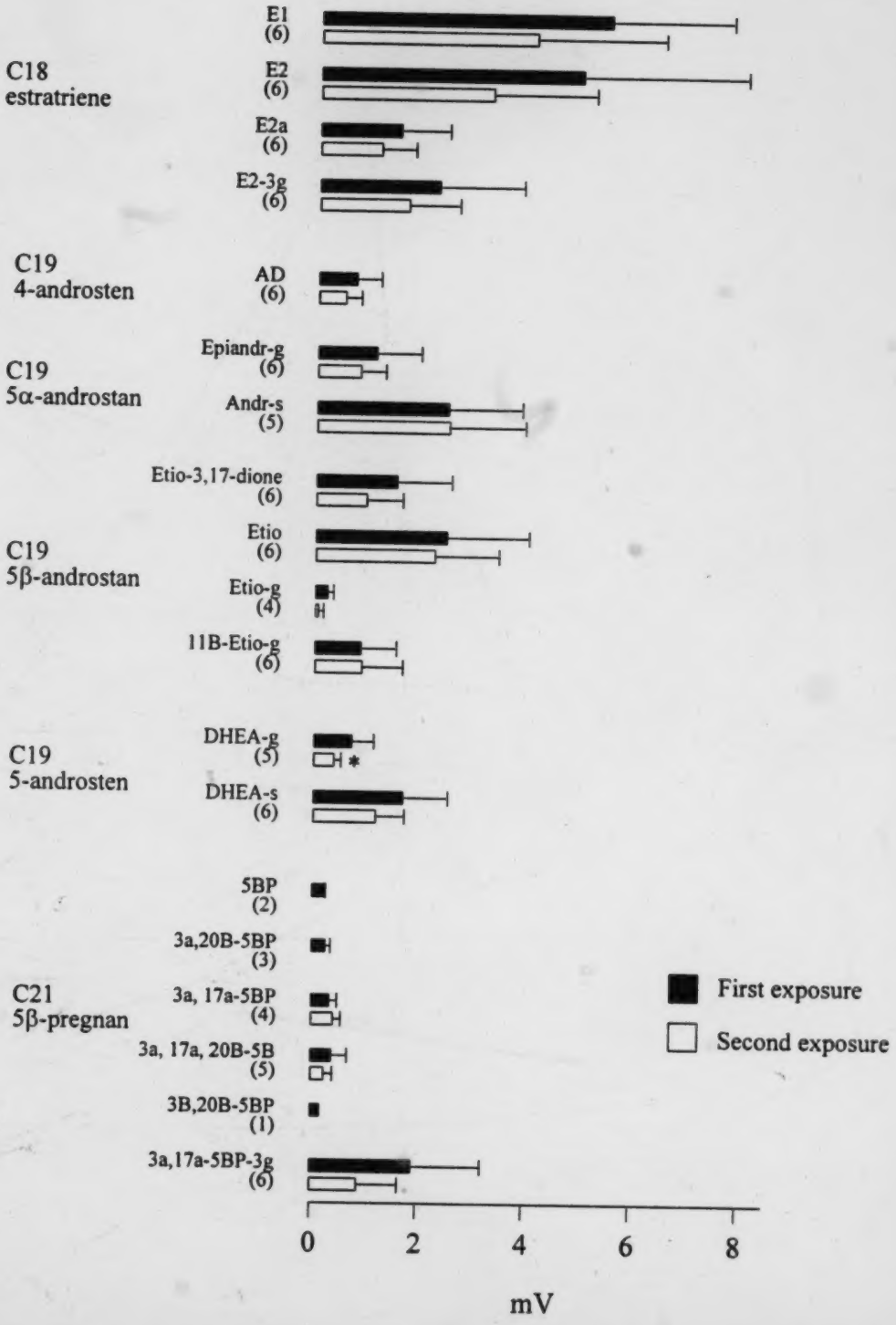
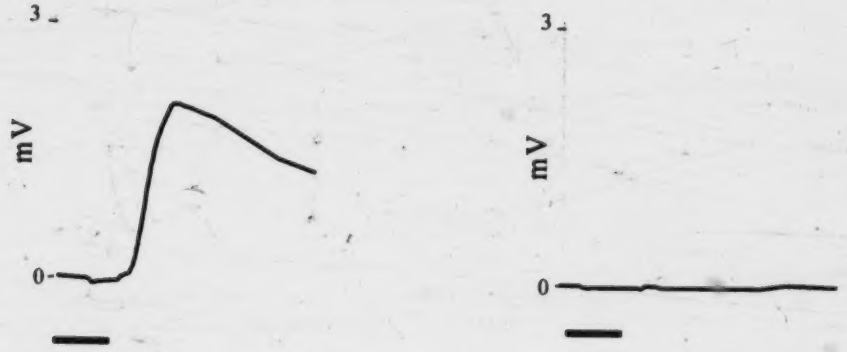

C18
estratriene
E1
(6)
E2
(6)
E2a
(6)
E2-3g
(6)
C19
4-androsten
AD
(6)
C19
5α-androstan
Epiandr-g
(6)
Andr-s
(5)
C19
5β-androstan
Etio-3,17-dione
(6)
Etio
(6)
Etio-g
(4)
11B-Etio-g
(6)
C19
5-androsten
DHEA-g
(5)
*
DHEA-s
(6)
C21
5β-pregnan
5BP
(2)
3a,20B-5BP
(3)
3a, 17a-5BP
(4)
3a, 17a, 20B-5B
(5)
3B,20B-5BP
(1)
3a,17a-5BP-3g
(6)
First exposure
Second exposure
0
2
4
6
8
mV

Figure 3.6. Effect of adaptation to 10^{-7} M estrone on EOG response to estradiol and estradiol-3-glucuronide.

Estradiol



Estradiol-3-glucuronide

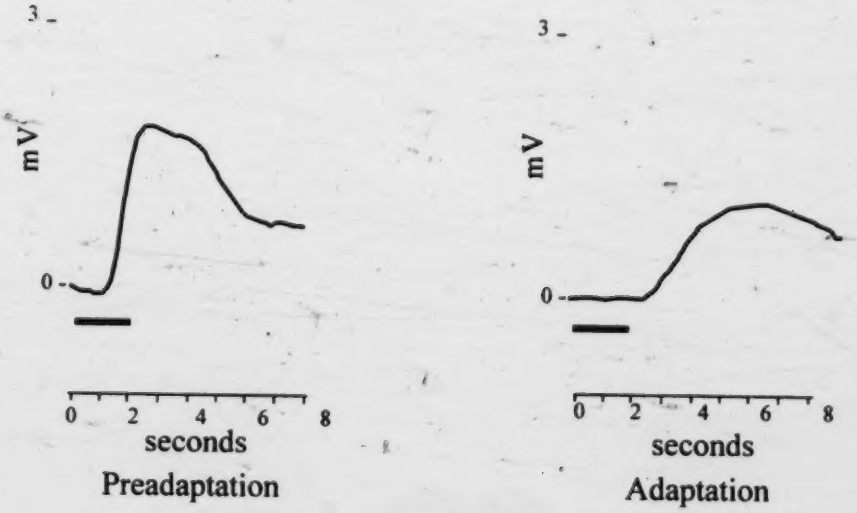


Figure 3.7. EOG response (mV; mean + s.d.) to 10^{-8} M steroids before and during adaptation to A) 10^{-7} M estrone, B) 10^{-7} M estradiol-glucuronide, and C) 10^{-7} M dehydroepiandrosterone-3-glucuronide. Sample size indicated beside bars. See Table 3.1 for full names of test steroids. * = response was reduced significantly ($P<0.05$) during adaptation.

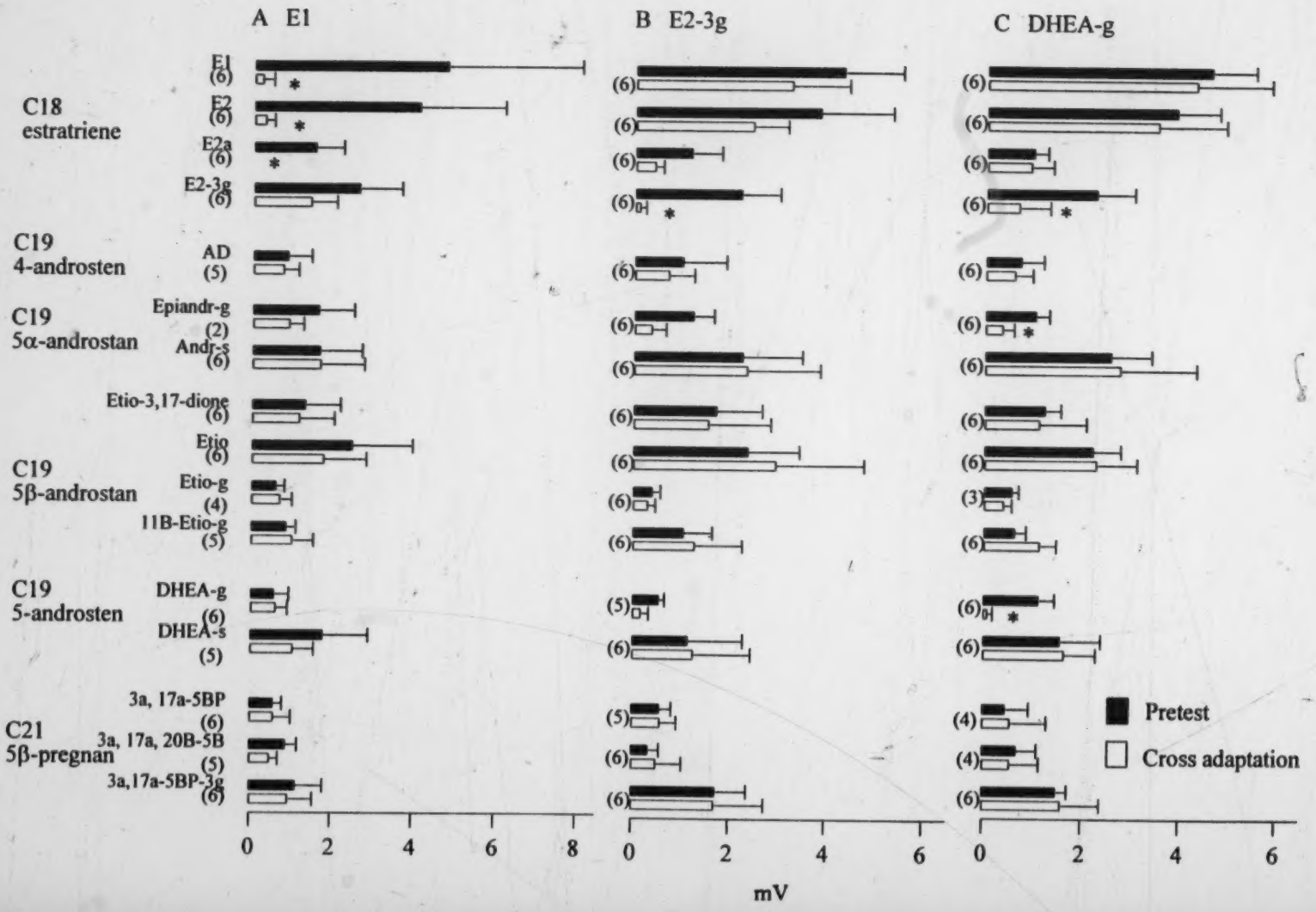
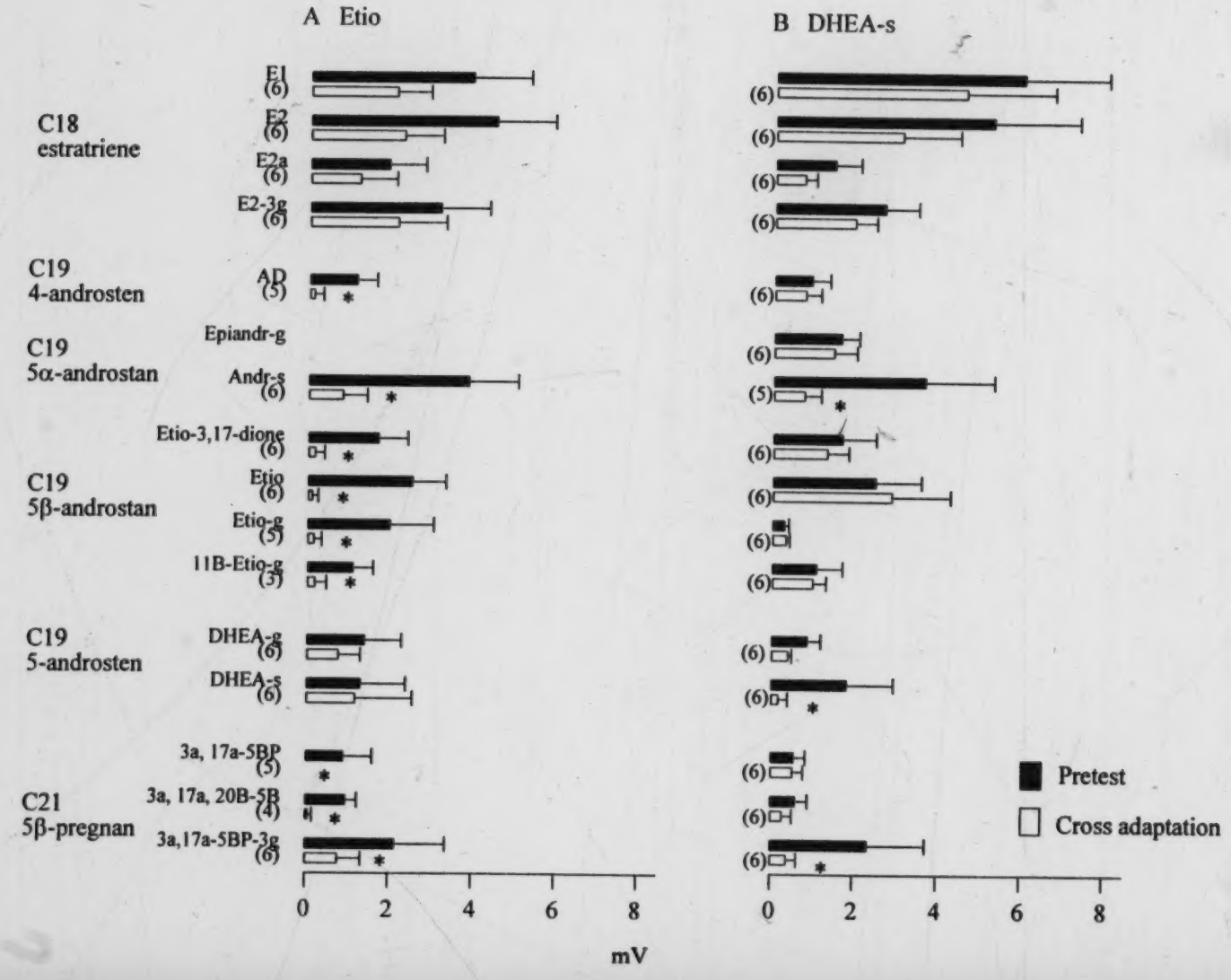

A E1
B E2-3g
C DHEA-g
C18 estratriene
C19 4-androsten
C19 5α-androstan
C19 5β-androstan
C19 5-androsten
C21 5β-pregnan
E1 (6)
E2 (6)
E2a (6)
E2-3g (6)
AD (5)
Epiandr-g (2)
Andr-s (6)
Etio-3,17-dione (6)
Etio (6)
Etio-g (4)
11B-Etio-g (5)
DHEA-g (6)
DHEA-s (5)
3a, 17a-5BP (6)
3a, 17a, 20B-5B (5)
3a,17a-5BP-3g (6)
Pretest
Cross adaptation
0 2 4 6 8
0 2 4 6
0 2 4 6
mV

Figure 3.8. EOG response (mV; mean + s.d.) to 10^{-8} M steroids before and during adaptation to A) 10^{-7} M etiocholanolone and B) 10^{-7} M dehydroepiandrosterone-sulphate. Sample size indicated beside bars. See Table 3.1 for full names of test steroids. * = response was reduced significantly ($P<0.05$) during adaptation.


A Etio
B DHEA-s
C18
estratriene
C19
4-androsten
C19
5α-androstan
C19
5β-androstan
C19
5-androsten
C21
5β-pregnan
E1
E2
E2a
E2-3g
AD
Epiandr-g
Andr-s
Etio-3,17-dione
Etio
Etio-g
11B-Etio-g
DHEA-g
DHEA-s
3a, 17a-5BP
3a, 17a, 20B-5B
3a,17a-5BP-3g
(6)
(5)
(4)
0
2
4
6
8
mV
Pretest
Cross adaptation

4. BEHAVIOURAL RESPONSE TO PUTATIVE STEROIDAL PHEROMONES

4.1. METHODS

4.1.1. Behavioural repertoire

To determine if the detected steroids reported in Chapter 3 induce behavioural responses, I observed and videotaped round goby behaviour to identify components that could be used to assess behavioural responses to water-borne steroids and other odours.

4.1.1.1. Spontaneous behaviours observed in the laboratory

Although I did not succeed in developing techniques that would ensure predictable spawning under the laboratory conditions described in Chapter 2, spontaneous spawning occurred occasionally throughout the year, and resulted in fertile, cone-shaped eggs being attached in a single layer to the ceiling of the male's pipe nest, as has been described in other gobiid species (Tavolga, 1954; Breder and Rosen, 1966; Senta and Wada, 1969; Cole, 1982; Ota *et al.*, 1996) Although spawning activities were never observed, casual observations and videotapes of both isolated and grouped gobies have identified a number of behaviours that appear to be useful candidates for assessing behavioural response to pheromones.

When held in isolation, both males and females used the PVC pipes for shelter. However, when kept in mixed-sex groups of 3-5 individuals, males excluded females from the pipes. Males defended acquired pipes by lunging after individuals that swam or rested within a few centimetres of the pipe. Often, a goby within a pipe would exhibit

coughing (see below). If there were more pipes than individuals within an aquarium, males used the pipes, and females sometimes used the pipes or rested outside and within the shadow of a pipe.

When held individually, males and females exhibit a similar pale, mottled colouration, and are difficult to sex without examining the urogenital papilla. However, when kept in aquaria containing females, males periodically turn completely black. Even the fins, which are normally a yellowish-green colour, also darken. The socially-induced black colour pattern appears to be restricted to males. For example, when 4 males and 4 females were anesthetized in 2-PE (0.05%) to induce melanophore expansion (see Tavolga, 1956), all 4 anesthetized males developed the black colouration within 30 min, whereas females darkened only slightly and their fins retained the normal yellowish-green colour. The black colouration exhibited by males kept in mixed-sex groups is presumed to function in courtship display, as colour changes have been reported in courting males of other goby species.

Much of the behaviour displayed by *Neogobius* is similar to behaviour described for *Bathygobius soporator* (Tavolga, 1954) and can be considered to be of three basic types: 1) behaviours potentially relevant to reproduction; 2) feeding behaviours; 3) locomotory behaviours. Each behaviour was monitored as an occurrence, and no attempt was made to assign intensity estimates or record duration.

4.1.1.2. Behaviours of potential reproductive significance

The following three behaviours (*gape*, *head lift*, and *cough*) involve head lifting and/or sound production.

Gape - As previously described for *B. soporator* (Tavolga, 1954), *gape* is displayed by both sexes, and consists of lifting the head, bending of the trunk, and inflation of the pharynx while the mouth and opercula gape widely (Tavolga, 1954). Unlike the situation in *Bathygobius*, *gaping* in *Neogobius* does not seem to be used in agonistic situations, because *gaping* is observed in isolated males and females. In a social aquarium, performance of *gape* by one fish is sometimes followed by *gaping* and other behaviours (*hop, fan*) in tankmates, although whether this indicates an agonistic interaction is not clear. When a hydrophone (ITC 6050C) was placed in the aquarium, it was found that *gaping* coincides with production of a non-harmonic sound (Appendix B), similar in frequency and amplitude to sounds recorded from *B. soporator* (Tavolga, 1958).

Head Lift - In this behaviour, exhibited by both males and females, the pelvic disc is positioned vertically so that the goby rests on the edge of the fin, and the trunk is bent upwards. *Head lift* may indicate alertness or odour detection. No sound is made during *head lift*.

Cough - *Coughing* was displayed by both males and females, usually when in the PVC tube, and occurred both in isolated and grouped fish. A *cough* is a series of openings and closings of the mouth in rapid succession. This behaviour, which differs from *gaping* because the head is not lifted, also is associated with sound production (Appendix C). The function of the *cough* is unknown, but could be a defensive or advertising behaviour.

The following behaviours are structurally unrelated, but might be expected to increase or decrease in response to a sex pheromone.

Fanning - *Fanning* is frequently observed in both sexes. Essentially, the fish anchors

itself to the substrate with its pelvic disc, and then waves its pectoral fins and body, and a sinusoidal wave passes down the dorsal fin (Tavolga, 1954). *Fanning* predominantly occurred while the fish was inside a PVC tube, perhaps in preparation for building a nest, or to produce a current of water through the tube.

Roll over - When in PVC tubes in social aquaria, males were observed to *roll over* and females were presumed to *roll over* when ovipositing eggs, since fertilized eggs were found on the ceiling of PVC tubes. *Roll over* is associated with gamete deposition in other gobiids (Tavolga, 1954; Senta and Wada, 1969; Cole, 1982; Ota *et al.*, 1996). Following *roll over*, gobies will cling with their pelvic disc to the top of a tube. Once upside down, males rub their urogenital papilla on the substrate, either in preparation for a female oviposition (Ota *et al.*, 1996), or in association with sperm release (Tavolga, 1954; Ota *et al.*, 1996). Videotapes of social aquaria showed that male *Neogobius* which had developed the black colouration periodically *roll over* and rub their ventral surface over the ceiling of the PVC tube. In other gobiid species, females will *roll over* in a nest tube to oviposit (Breder and Rosen, 1966; Miller, 1984)

4.1.1.3. Feeding behaviours

Gobies occasionally snapped at particles in the water column, or picked up gravel in the mouth, manipulated it for several seconds, and then spat it out. Both behaviours were categorized as *feeds* and were not differentiated. The spitting of ingested gravel is not the same as the scooping/nest preparation behaviour seen in *B. soporator* (Tavolga, 1954), as the round goby does not transport the gravel to other parts of the aquarium. In natural settings, males typically construct nests under rocks and logs, or in any suitable

cavity (Charlebois *et al*., 1997), and will colonize nests in both cobble and sandy habitats (MacInnis and Corkum, in press).

4.1.1.4. Behavioural measures of activity

Changes in the following behaviours might indicate detection of an odour:

Movement - To determine general activity, the 90 cm length of observation aquaria was divided into three equal sections by vertical lines and the number of times a fish's head crossed a line was recorded.

Hop - A *hop* is a short forward movement, typically less than one body length and is accomplished by a fanning of the pectoral fins and a propulsive thrust from the pelvic disc. Because males and females usually moved about the aquaria using short hopping motions, an increase in frequency might indicate the goby is searching for an odour source.

Entry/Exit - The number of times a fish entered or left its nest tube was recorded as a measure of nest vigilance and was expected to change if the fish received sexual or agonistic cues.

Ventilation rate - *Ventilation rate* (rate of opercular openings) was used as a measure of odour detection because, in some benthic fish, water flow through the naris changes in synchrony with ventilation (Nevitt, 1991). Thus, ventilation rate might be expected to increase in response to an odour.

4.1.2. Behavioural responses to detected steroids

Once detected steroids (steroids that elicited an EOG response) had been assigned

to receptor classes by EOG cross-adaptation studies (Section 3.2.3.), I then determined if steroids from each of the four receptor classes could induce behavioural changes in male and female gobies.

4.1.2.1. Testing apparatus

Single fish were placed in aerated 100 L test aquaria and allowed to acclimate for at least five days before testing. Test aquaria were opaque on three sides, with one lateral side left clear for viewing, and were provided with gravel substrate and one piece of PVC pipe (6 cm diameter, 12 cm length) placed with one end facing the clear viewing side. To minimize disturbance during observations, fish were observed through a small hole cut in an opaque cloth curtain. Steroid solutions or ethanol vehicle were held in 10 ml plastic syringes and injected into the test aquaria through silastic tubing which was fed through a hole in the cloth curtain and anchored to each aquarium, with the free end outside of the water and directly over the PVC tube. To determine how test odours might disperse, water flow was turned off and dye (malachite green) was injected into an aquarium; the dye plume reached the PVC tube within one min, and was dispersed evenly throughout the entire aquarium within 3 min.

4.1.2.2. Testing procedure

Water flow into an aquarium was turned off two hours before an experiment, the fish was tested with only one odour per day, and water flow was resumed to allow overnight flushing. The same fish was tested with different odours on consecutive days. In each test, a fish was observed for a twelve minute pretest period, a test solution was then injected, and the fish was observed for an additional 24 min. Ventilation was

measured for every third minute before and after addition of an odour. All other behaviours (Section 4.1.1) were counted continuously throughout the test. Six males and six females were tested with each odour.

4.1.2.3. Test odours

The most potent steroids for each of the four odour receptor classes (E1, E2-3g, Etio, and DHEA-s) were tested both individually and as a mixture. A few of the less potent steroids (Etio-g, Andr-s, and DHEA-g) also were tested individually to determine if steroids acting through the same odour class induced the same response(s). Control solutions for steroid odours included the ethanol vehicle used as solvent for all steroids, and a conjugated androgen (testosterone-3-glucuronide; Test-g) that did not induce an EOG response.

For all steroids tested individually, 1 ml of a 10^{-3} M steroid-ethanol solution was added to the aquarium to create a 10^{-8} M concentration after dispersal. For the ethanol control test, the amount of solvent in a 10^{-8} M steroid solution (1 ml ethanol) was added to the test aquarium. For the mixture of steroids (E1, E2-3g, Etio and DHEA-s), 100 µl of each steroid was taken from 10^{-3} M stock solutions, mixed together, and added to the observational aquarium to make the final concentration of each steroid 10^{-9} M. Only males (N=6) were tested with the mixture.

To determine if any behaviour change observed following the addition of a steroid to a test aquarium was a behaviour change specific to steroids, two non-steroidal odours also were tested: 10^{-6} M L-alanine and a food odour. Ten ml of a 10^{-2} M L-alanine aqueous solution were added to the aquarium to create a 10^{-6} M final concentration. Food

odour was prepared by thawing 1 g of frozen brine shrimp in 100 ml of distilled water, allowing the stirred mixture to settle, and injecting 10 ml of supernatant fluid to test aquaria.

4.1.2.4. Data analysis

To determine if odour addition affected behaviour, total occurrences of each behaviour in the 12 min pretest were compared to total occurrences in each of the two 12 min post-stimulus intervals using paired Wilcoxon tests, with a Bonferroni adjustment ($P<0.025$) (Systat, 1994)

Ventilation rate data were transformed into percent changes by expressing response to odours as a percentage of the mean pretest value (basal ventilation rate; mean of the four 1-minute samples prior to exposure). Based on observed increases in ventilation rate, it appeared to take three min or less for the goby to detect an odour. To determine if the change in ventilation rate after the addition of a compound into the water was significant, the mean of the ventilation rates observed at 4 min and 7 min were compared to the mean pretest value using a Wilcoxon paired test for non-parametric data (Systat, 1994).

To determine if the duration of the ventilation response changes with exposure to different compounds, the mean ventilation rate for the 12 min pretest was compared to mean ventilation rate observed between 13-24 mins using Wilcoxon paired test for non-parametric data (Systat, 1994).

To determine if the basal ventilation rate of males and females differed, the mean pretest ventilation rates for all testing compounds (E1, E2-3g, Etio, Etio-g, Andr-s,

DHEA-s, DHEA-g, EtOH and Test-g) were compared between sexes using a non-paired Student's T-test (Systat, 1994).

4.1.3. Behavioural concentration-response studies

Steroids that induced a behavioural change when tested at 10^{-8} M (section 4.1.2.) (E1, E2-3g and Etio on male fish) were used in a concentration response experiment to determine the threshold concentration for behavioural response and whether behaviours change quantitatively at suprathreshold concentration. The protocol was identical to that described in section 4.1.2., except that the final concentrations in the test aquaria ranged from 10^{-12} M to 10^{-8} M, and were prepared by diluting appropriate volumes of 10^{-3} M steroid solutions into 100 ml of double-distilled, de-ionized water, and then adding 1 ml of this solution to the test aquarium. For example, to make a final concentration of 10^{-11} M E1, 100 µl of 10^{-3} M E1 stock solution (section 3.1.2) was added to 100 ml of double-distilled, de-ionized water, and then 1 ml of the resulting 10^{-6} M E1 solution was added to the test aquarium. Testing of individual concentrations began at 10^{-12} M, and increased in log molar increments on consecutive days.

Analysis of the concentration response studies was similar to that described in section 4.1.2.4. Also, to determine if ventilation rate increases observed at suprathreshold concentrations increased in magnitude as the concentration increased, the mean ventilation rate increases (4th and 7th minute post-stimulus) were compared for each concentration using a Kruskal Wallis nonparametric ANOVA.

To determine if there was a significant correlation between pre-stimulus and post-

stimulus ventilation rates, the mean pretest ventilation rate for males exposed to E1 at 10^{-10} M, 10^{-9} M, and 10^{-8} M was compared first to the mean ventilation rate observed at 4 min and 7 min using Spearman's rank correlation (Systat, 1994). Then the mean pretest ventilation rate was compared to the mean percent increase in ventilation rate observed for 4th and 7th minutes post stimulus using a Spearman's rank correlation for non-parametric data (Systat, 1994).

4.1.4. Behavioural cross-adaptation

To determine if *Neogobius* can behaviourally discriminate steroids that act on different classes of olfactory receptors, an experiment using ventilation as the behavioural response was designed to mimic the EOG cross-adaptation experiment. The experiment tested two hypotheses: 1) that steroids detected on the same receptor mechanism can not be discriminated behaviourally; 2) that steroids detected via separate receptor mechanisms can be discriminated.

For the pretest, the protocol was similar to that described in 4.1.2., in that it used the four test steroids (E1, E2-3g, Etio, and DHEA-s) representing the four proposed receptor classes. In addition, the pretest of this experiment also used E2 and Andr-s, which induce large EOG responses, and evidently act via the E1 and Etio receptors respectively. The time between pretests and behavioural cross-adaptation tests was approximately 3 months.

The protocol for the behavioural cross-adaptation was also similar to that described in section 4.1.2. except that a fish was exposed sequentially to two steroids.

First, an *adapting* steroid (either 10^{-8} M E1 or Etio) was injected after the pretest observational period and the fish observed for 24 min. Then a *test* steroid (10^{-9} M) was injected, and the fish observed for a further 24 min. Behavioural data were collected in the same way as that described in 4.1.2. and six male fish were tested in each treatment group.

E1 and Etio, the most potent steroids for the two receptor classes that induced a behaviour change, were used as adapting steroids. The test steroids were either: the adapting steroid (used as a control treatment); a steroid believed to be acting via the same receptor class (E2 and Andr-s), or a steroid believed to be acting via a separate receptor mechanism (Etio, E1 and E2-3g). A change in ventilation was expected after the first exposure to the adapting steroid. A second change in behaviour was expected only if the second odour acted on a different receptor class.

Ventilation was measured and analyzed as in section 4.1.2.4. All other behaviours were summed for 12 min time intervals for each behaviour type. The behaviour that occurred in the 12 min pretest was compared to the behaviour occurring in the two 12 min time intervals following the addition of each steroid using a Kruskal Wallis ANOVA for non-parametric data (Systat, 1994).

4.2. RESULTS

Throughout the observation period for all experiments, gobies performed most of the behaviour described in section 4.1.1. However, the only measure that changed consistently after the addition of an olfactory stimulant was ventilation.

4.2.1. Behavioural responses to detected steroids

Ethanol solvent did not elicit any change in the ventilation rate in either males or females (Fig. 4.1a). Test-g (10^{-8} M), which does not induce an EOG response, also did not induce any change in the ventilation rate (Fig. 4.1b).

Changes in ventilation rate after addition of a detected steroid were observed in both male and female fish; however, sexual dimorphism was apparent in the response. The estrogen compounds, E1 and E2-3g, induced a significant increase in the ventilation rates of males, but did not affect females (Fig. 4.2). In contrast, Etio significantly increased ventilation rates in both males and females (Fig. 4.3a). Similarly, Etio-g and Andr-s, steroids believed to be acting via the same receptor mechanism as Etio, also increased ventilation in both males and females (Fig. 4.3b, c). DHEA-s and DHEA-g did not significantly change ventilation rates of either sex (Fig. 4.4), although the variance increased after exposure to DHEA-g due to a slight increase in ventilation in one fish in each treatment group (Fig 4.4b).

The mixture of 10^{-9} M steroids (E1, E2-3g, Etio, and DHEA-s) induced an immediate increase in ventilation rate (Fig. 4.4c). The mix of steroids did not change any

other behaviour described in section 4.1.1. (data not shown).

The duration of the ventilation response was influenced both by gender and the steroid tested. Males had longer ventilation rate increases in response to E1 and Etio than shown to E2-3-g, while females had a prolonged response to Etio-g and Andr-s (Table 4.1). Basal ventilation rates of males (33.9±3.4 (mean±s.d); N=54) and females (32.0±2.3; N=54) were not significantly different (P>0.05).

L-alanine (10^{-6} M) did not induce any change in the ventilation rate of males or females (Fig. 4.5). Although food odour increased ventilation rates of both sexes, the change was significant only in males (Fig. 4.5). Food odour also significantly increased feeding behaviours, picking at gravel, and snapping at particles in the water column (Fig. 4.6a). These behaviours were not affected by L-alanine or ethanol (Fig. 4.6b, c), or by any steroid (data not shown).

4.2.2. Behavioural concentration-response studies

The threshold of the ventilation rate response was 10^{-10} M for E1 and Etio, and 10^{-9} M for E2-3g (Fig. 4.7). Once threshold had been reached, ventilation rate did not significantly increase in magnitude at higher odour concentrations.

Prestimulus ventilation rates were not significantly correlated to post-stimulus ventilation rates for males exposed to E1 at 10^{-10} M, 10^{-9} M, and 10^{-8} M (Spearman's rank correlation coefficient = 0.124; P=0.6247) (Fig 4.8a). However, prestimulus ventilation rates were significantly correlated with percent ventilation rate increase (Spearman's rank correlation coefficient = -0.587; P=0.0104) (Fig 4.8b).

4.2.3. Behavioural cross-adaptation

Andr-s and E2 are believed to act via different receptor mechanisms; Andr-s via the Etio and DHEA-s receptor mechanisms, and E2 via the E1 receptor mechanism. Both Andr-s and E2 significantly increased ventilation rate when injected to aquaria to create a final concentration of 10^{-9} M (Fig. 4.9a, b). These steroids were used in the subsequent behavioural cross-adaptation study.

In behavioural cross-adaptation experiments using 10^{-8} M E1 as the adapting steroid, males significantly increased ventilation rate when exposed to the adapting stimulus (Fig. 4.10). However, ventilation was unaffected by subsequent addition of either 10^{-9} M E1 or 10^{-9} M E2 (Fig. 4.10a, b). In contrast, ventilation rate of males adapted to 10^{-8} M E1 increased significantly in response to subsequent addition of 10^{-9} M Etio or 10^{-9} M E2-3g, both believed to act via separate receptor mechanisms (Fig. 4.10c, d)

Results of behavioural cross-adaptation studies using Etio as the adapting steroid were similar to those using E1. 10^{-8} M Etio (adapting stimulus) significantly increased ventilation (Fig. 4.11), but ventilation of adapted fish was unaffected by subsequent exposure to either 10^{-9} M Etio or 10^{-9} M Andr-s, both thought to act via the Etio receptor. In contrast, 10^{-9} M steroids (E1, E2-3g) thought to act through other receptors significantly increased ventilation in Etio-adapted fish (Fig 4.11c, d).

DHEA-s did not increase ventilation rate in male gobies (section 4.2.1). However, the EOG cross-adaptation revealed that two steroids that act via the same receptor as DHEA-s may also interact with the Etio receptor, and that one of those, Andr-

s. does increase ventilation rate in male gobies. When 10^{-8} M DHEA-s was used as an adapting stimulus, the ventilation rate remained unchanged (Fig. 4.12). If 10^{-9} M Andr-s then was added as the test stimulus, there was a slight, but insignificant increase in ventilation rate (Fig. 4.12).

During the course of the entire behavioural study, changes in hopping behaviour of gobies were observed during only two behavioural cross-adaptation tests. When E1 was used as an adapting solution, males significantly increased the number of hops when either E2 or E2-3g was added after an initial E1 exposure (Fig. 4.13b, d).

Table 4.1. Duration of ventilation rate response 13-24 mins after exposure to 10^{-8} M steroids. Responses that were still significant after 13-24 mins (Wilcoxon, $P<0.05$) indicate a prolonged ventilation rate increase.

	Males	**Females**
E1	0.027	-
E2-3g	n.s.	-
Etio	0.028	n.s.
Etio-g	n.s.	0.026
Andr-s	n.s.	0.027
mixture	n.s.	not tested

Figure 4.1. Percent change in basal ventilation rate (mean±s.d.) of male and female gobies after addition (arrow) of: A - 1 ml ethanol; B - 10^{-8} M testosterone-glucuronide. Ventilation rates in response to test substances were calculated as percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups.

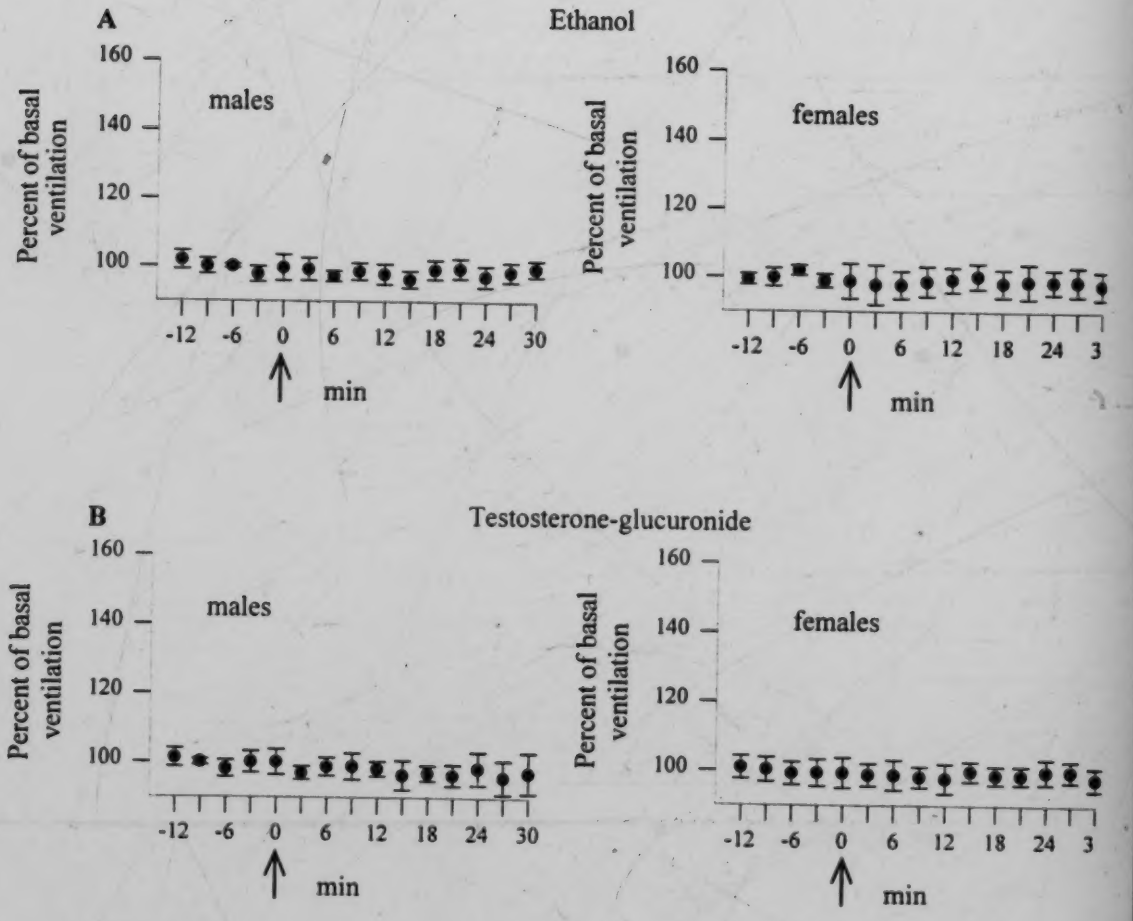

A
Ethanol
Percent of basal ventilation
160
140
120
100
males
females
-12
-6
0
6
12
18
24
30
min
B
Testosterone-glucuronide

Figure 4.2. Percent change in basal ventilation rate (mean±s.d.) of male and female gobies after addition of: A - 10^{-8} M E1; B - 10^{-8} M E2-3g. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). N=6 for all groups. *, mean ventilation rate measured of the 4th and 7th min is significantly different than mean pretest ventilation rate (P<0.05). ** (P<0.01)

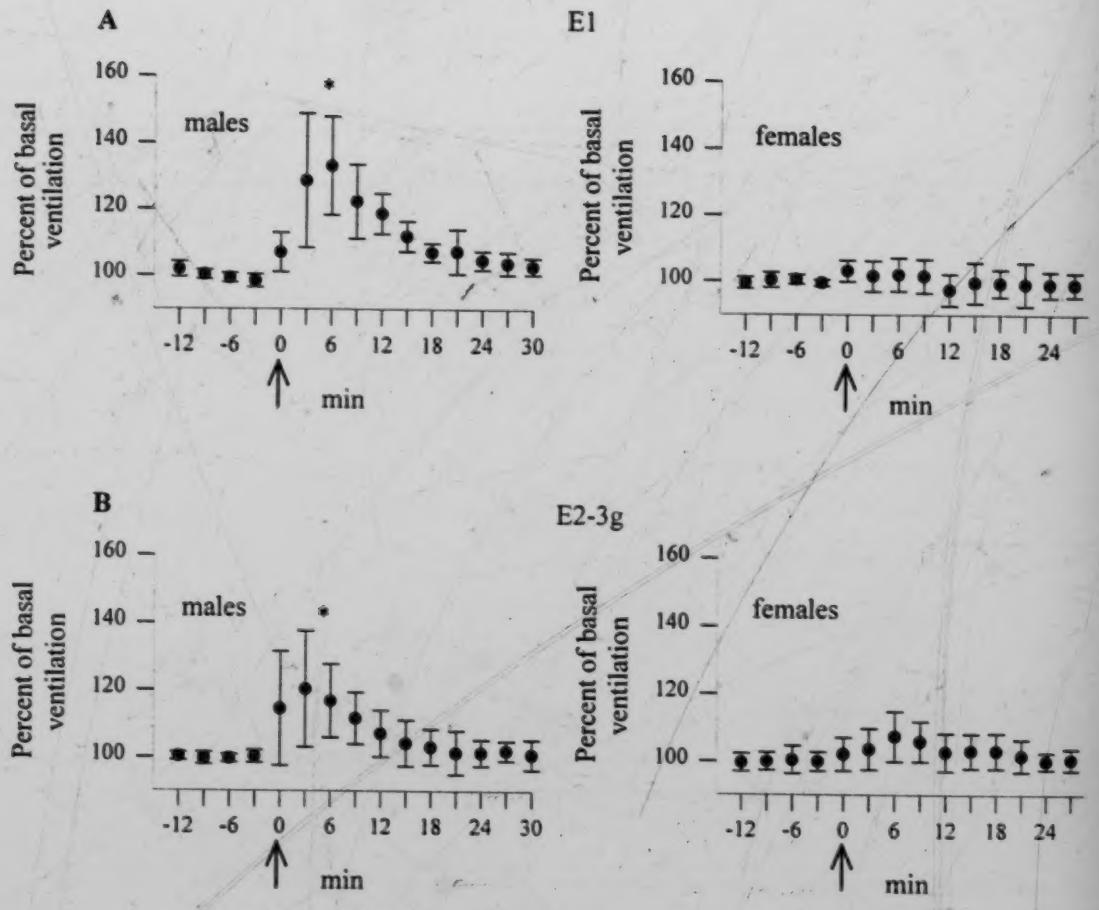

A
E1
B
E2-3g
males
females
Percent of basal ventilation
160
140
120
100
-12
-6
0
6
12
18
24
30
min
*

Figure 4.3. Percent change in basal ventilation rate (mean±s.d.) of male and female gobies after addition of: A - 10^{-8} M Etio; B - 10^{-8} M Etio-g; C - 10^{-8} M Andr-s. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

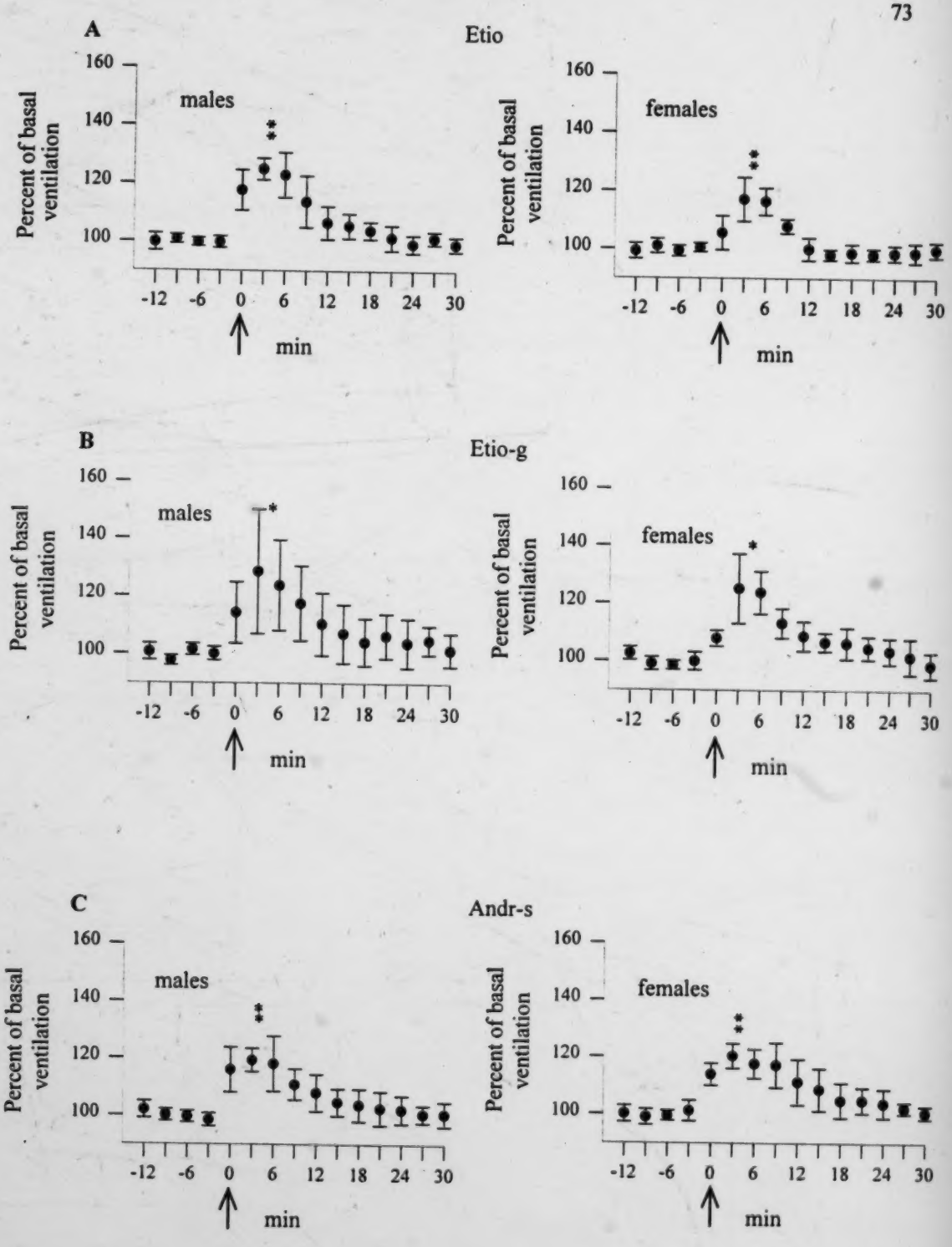

A
Etio
males
females
Percent of basal ventilation
160
140
120
100
-12
-6
0
6
12
18
24
30
min
B
Etio-g
males
females
C
Andr-s
males
females

Figure 4.4. Percent change in basal ventilation rate (mean±s.d.) of male and female gobies after addition of: A - 10^{-8} M DHEA-s; B - 10^{-8} M DHEA-g; C - 10^{-9} M steroid mixture (E1, E2-3g, Etio and DHEA-s). Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

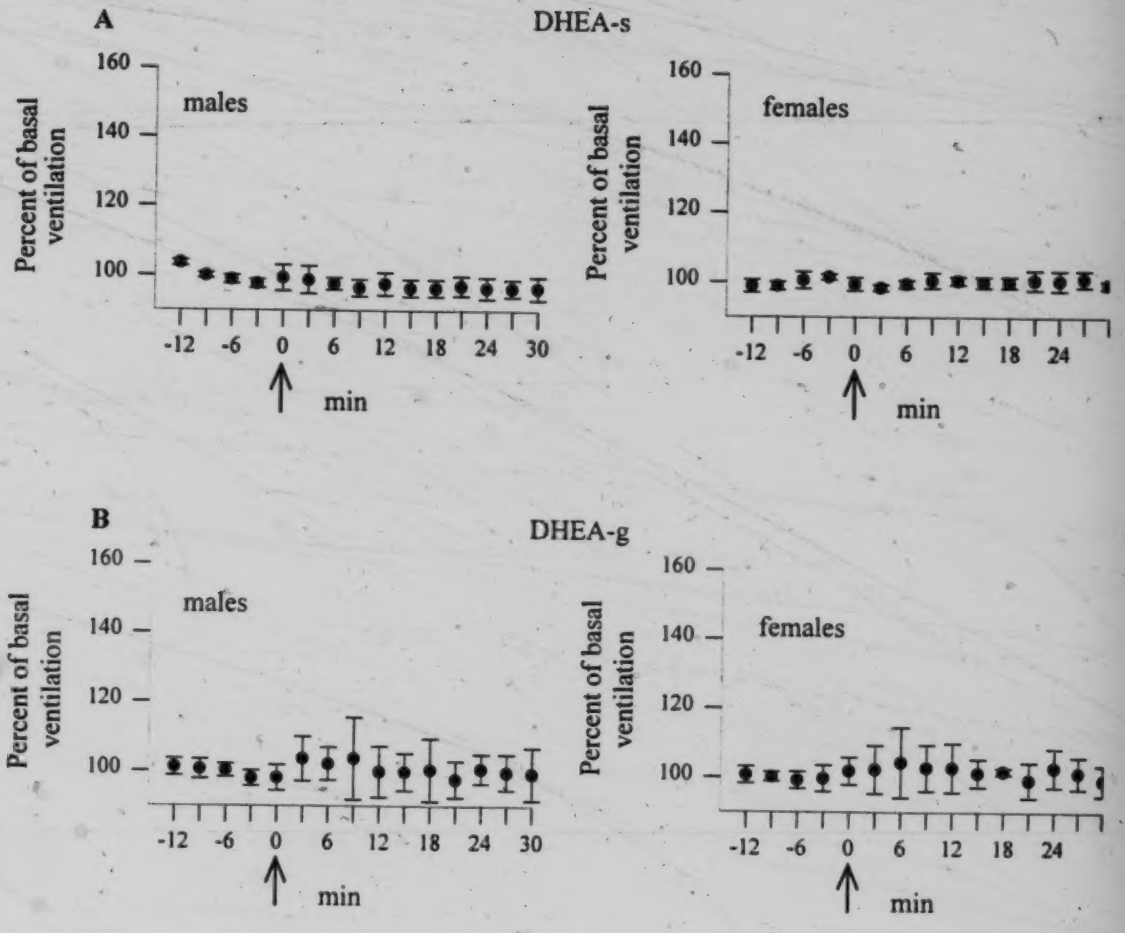

A
DHEA-s
males
females
Percent of basal ventilation
160
140
120
100
-12
-6
0
6
12
18
24
30
min
B
DHEA-g
males
females
Percent of basal ventilation
C
E1, E2-3g, Etio, DHEA-s mixture
males
Percent of basal ventilation
min

Figure 4.5. Percent change in basal ventilation rate (mean±s.d.) of male and female gobies after addition of: A - 10^{-6} M alanine; B - food odour. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

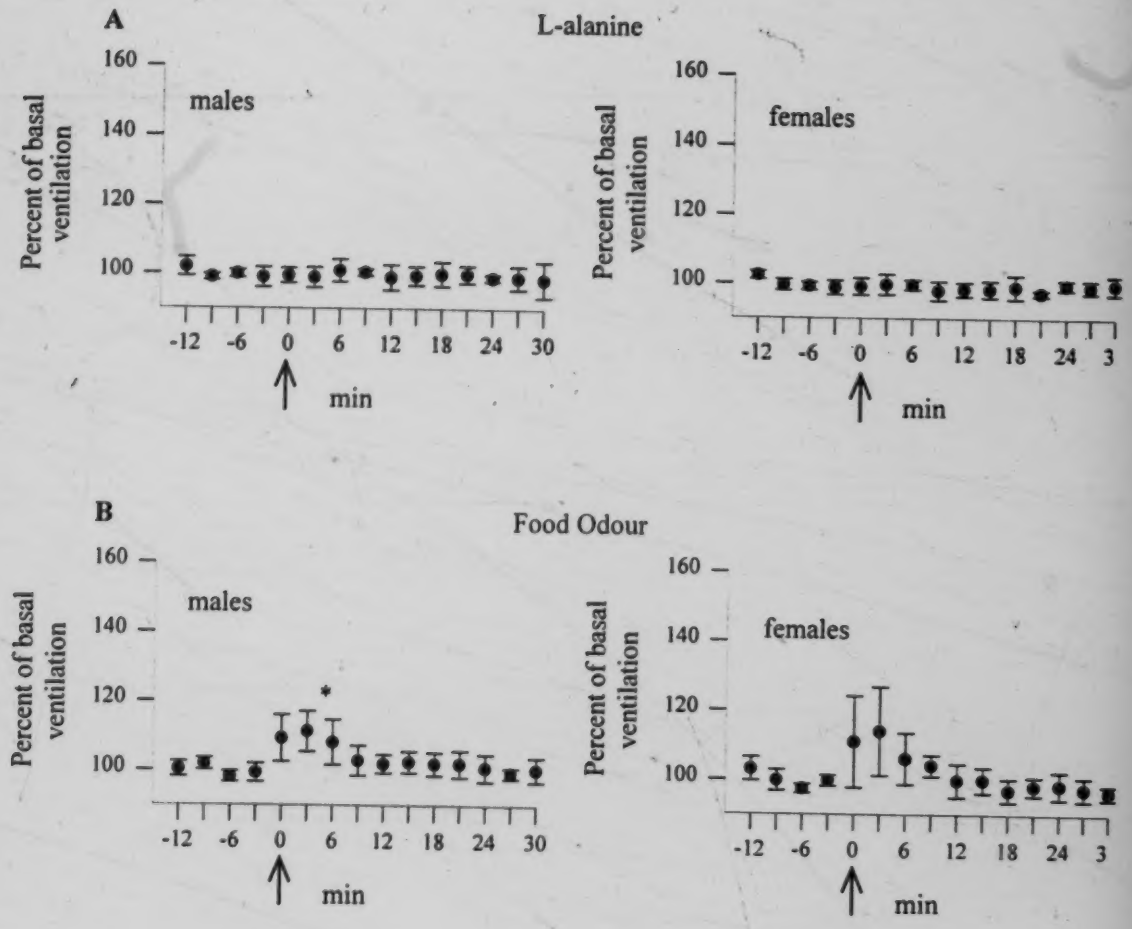
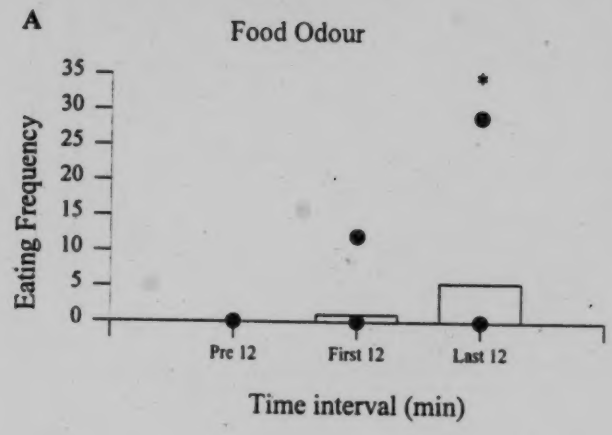
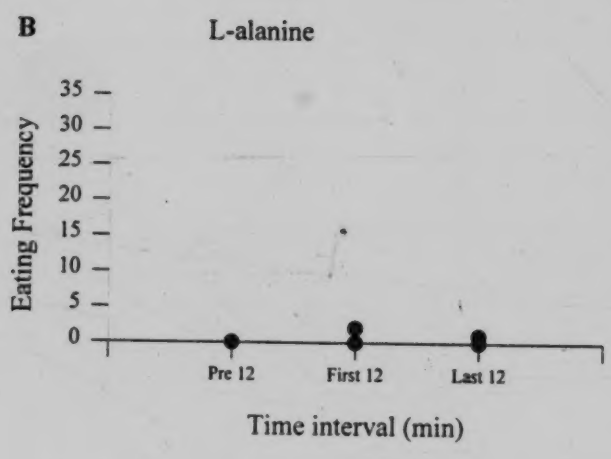

A
L-alanine
males
females
Percent of basal ventilation
160
140
120
100
-12
-6
0
6
12
18
24
30
min
B
Food Odour
*

Figure 4.6. Changes in eating frequency after addition of: A - food odour; B- 10^{-6} M alanine; C- ethanol. Eating frequency observed in the 12 min pretest is compared to the first 12 min following addition of a test compound, and the next 12 min time interval. Bars represent median values, points represent range. N=6 females in all groups. *median eating frequency is significantly different than eating frequency observed in the pretest (P<0.05).


A
Food Odour
Eating Frequency
35
30
25
20
15
10
5
0
Pre 12
First 12
Last 12
Time interval (min)


B
L-alanine
Eating Frequency
35
30
25
20
15
10
5
0
Pre 12
First 12
Last 12
Time interval (min)

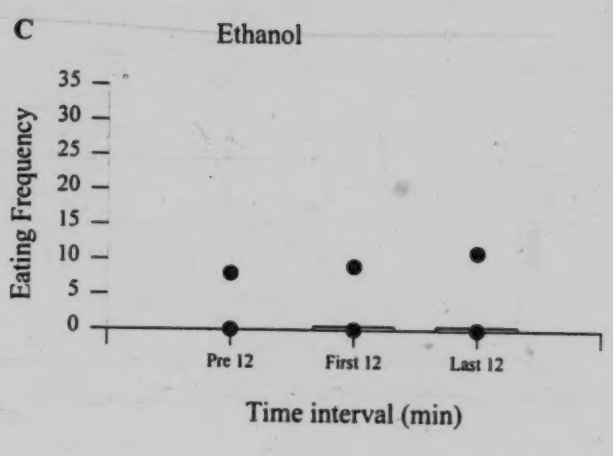

C
Ethanol
Eating Frequency
35
30
25
20
15
10
5
0
Pre 12
First 12
Last 12
Time interval (min)

Figure 4.7. Percent change in basal ventilation rate (mean±s.d.) of male gobies after addition of E1, E2-3g and Etio in concentration response studies. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

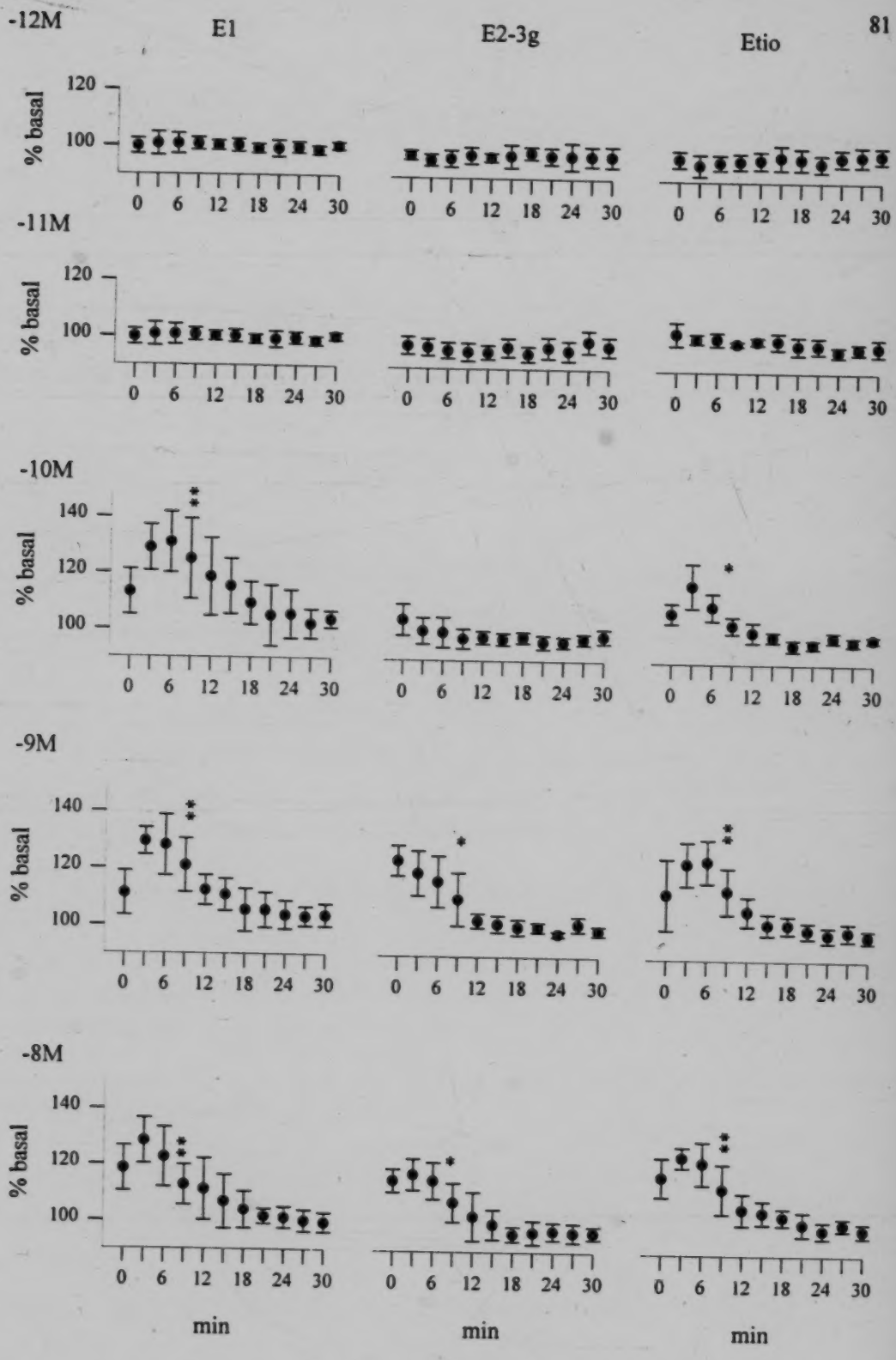
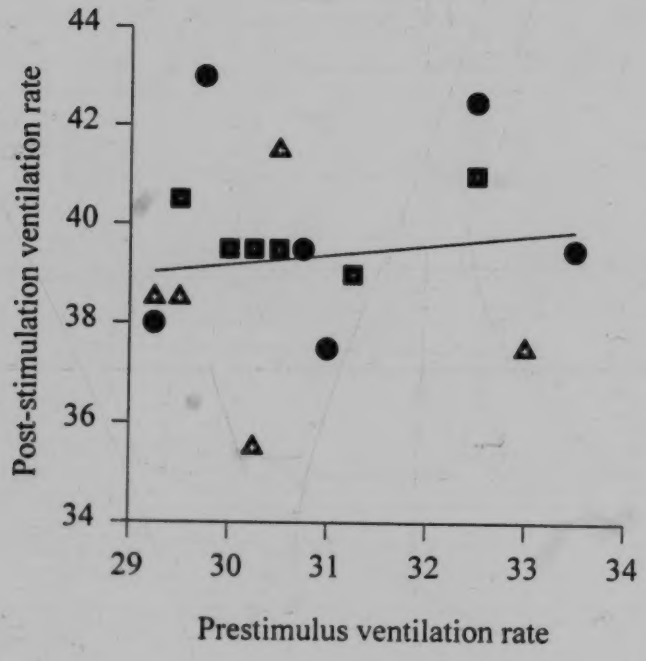

-12M
E1
E2-3g
Etio
-11M
-10M
-9M
-8M
% basal
120
100
140
0 6 12 18 24 30
min
*
**

Figure 4.8. Mean prestimulus ventilation rates in the 12 min preceding odour addition compared to: A - mean post-stimulus ventilation rates for the 4th and 7th min (Spearman's correlation, P=0.6247); B - mean percent change in ventilation rate for the 4th and 7th min post-stimulus (Spearman's correlation, P=0.0104). Stimuli are odour additions of 10^{-10} M E1 (●), 10^{-9} M E1(■) and 10^{-8} M E1(▲).

A


Post-stimulation ventilation rate
44
42
40
38
36
34
29
30
31
32
33
34
Prestimulus ventilation rate

B

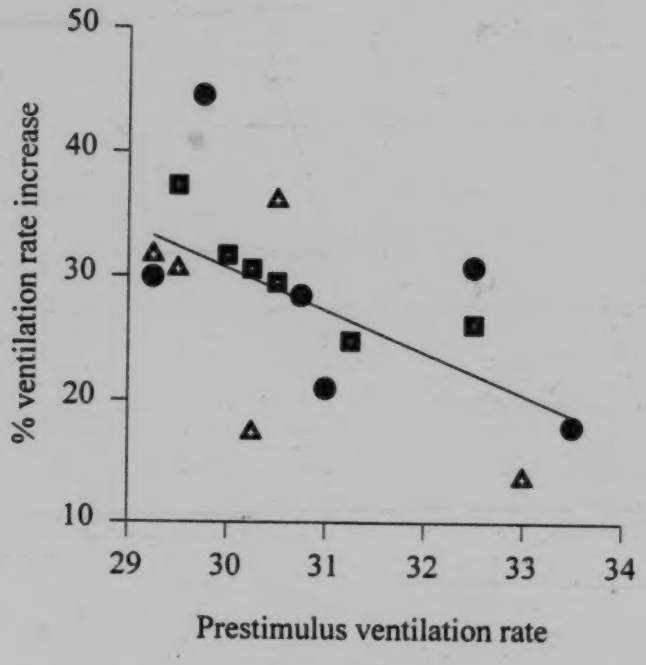
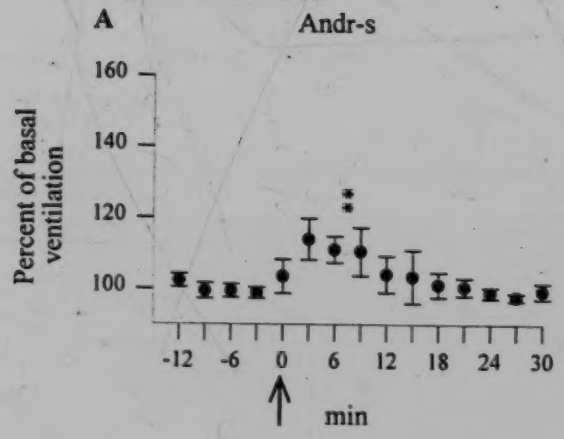

% ventilation rate increase
50
40
30
20
10
29
30
31
32
33
34
Prestimulus ventilation rate

Figure 4.9. Percent change in basal ventilation rate (mean±s.d.) of male gobies after addition of: A - 10^{-9} M Andr-s; B - 10^{-9} M E2. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.


A
Andr-s
Percent of basal ventilation
160
140
120
100
**
-12
-6
0
6
12
18
24
30
min

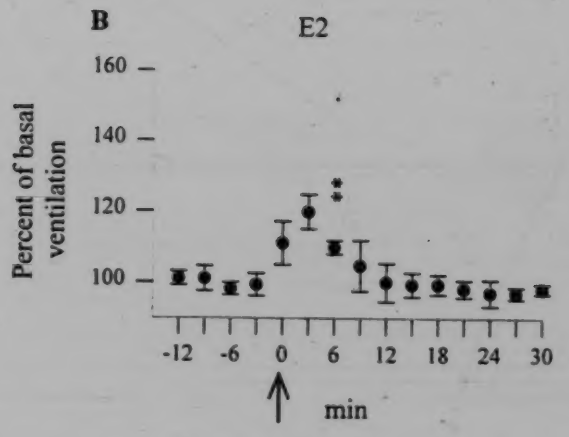

B
E2
Percent of basal ventilation
160
140
120
100
**
-12
-6
0
6
12
18
24
30
min

Figure 4.10. Percent change in basal ventilation rate (mean±s.d.) of males in behavioural cross-adaptation studies using 10^{-8} M E1 as the adapting steroid. In all cases, E1 was added to the aquarium first, after a 12 min pretest (as indicated by the first arrow). After monitoring ventilation rates for 24 min, a second steroid (A - 10^{-9} M E1; B - 10^{-9} M E2; C - 10^{-9} M Etio; D- 10^{-9} M E2-3g) was added to the aquarium and fish were observed for an additional 24 min. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding addition of first steroid). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

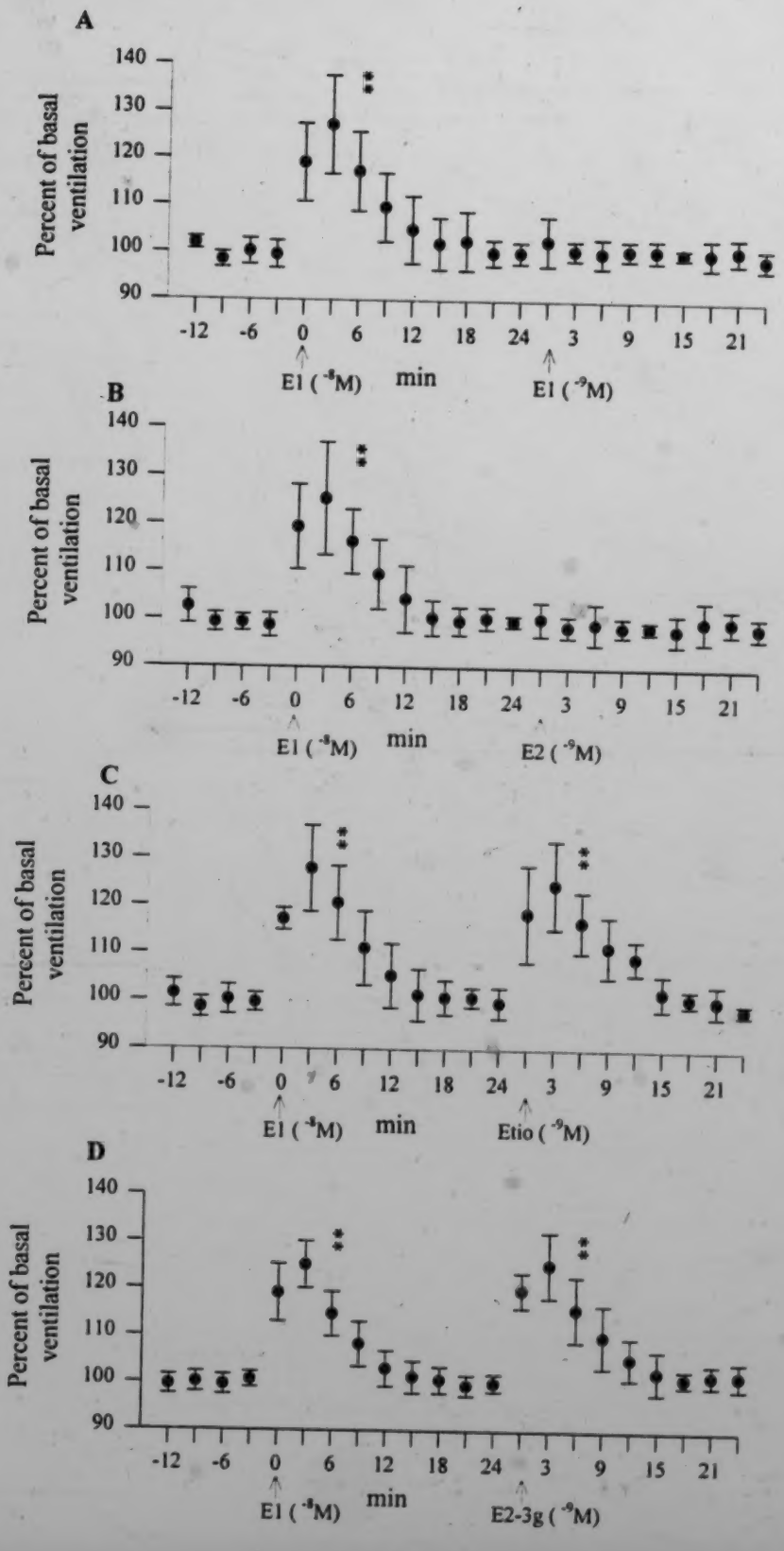

A
Percent of basal ventilation
140
130
120
110
100
90
-12 -6 0 6 12 18 24 3 9 15 21
E1 (-8M)
min
E1 (-9M)
B
Percent of basal ventilation
E1 (-8M)
min
E2 (-9M)
C
Percent of basal ventilation
E1 (-8M)
min
Etio (-9M)
D
Percent of basal ventilation
E1 (-8M)
min
E2-3g (-9M)

Figure 4.11. Percent change in basal ventilation rate (mean±s.d.) of males in behavioural cross-adaptation studies using 10^{-8} M Etio as the adapting steroid. In all cases, Etio was added to the aquarium first, after a 12 min pretest (as indicated by the first arrow). After monitoring ventilation rates for 24 min, a second steroid (A - 10^{-9} M Etio; B - 10^{-9} M Andr-s; C - 10^{-9} M E1; D- 10^{-9} M E2-3g) was added to the aquarium and fish were observed for an additional 24 min. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding addition of first steroid). *N*=6 for all groups. Statistical analysis of ventilation response was as described in Figure 4.2.

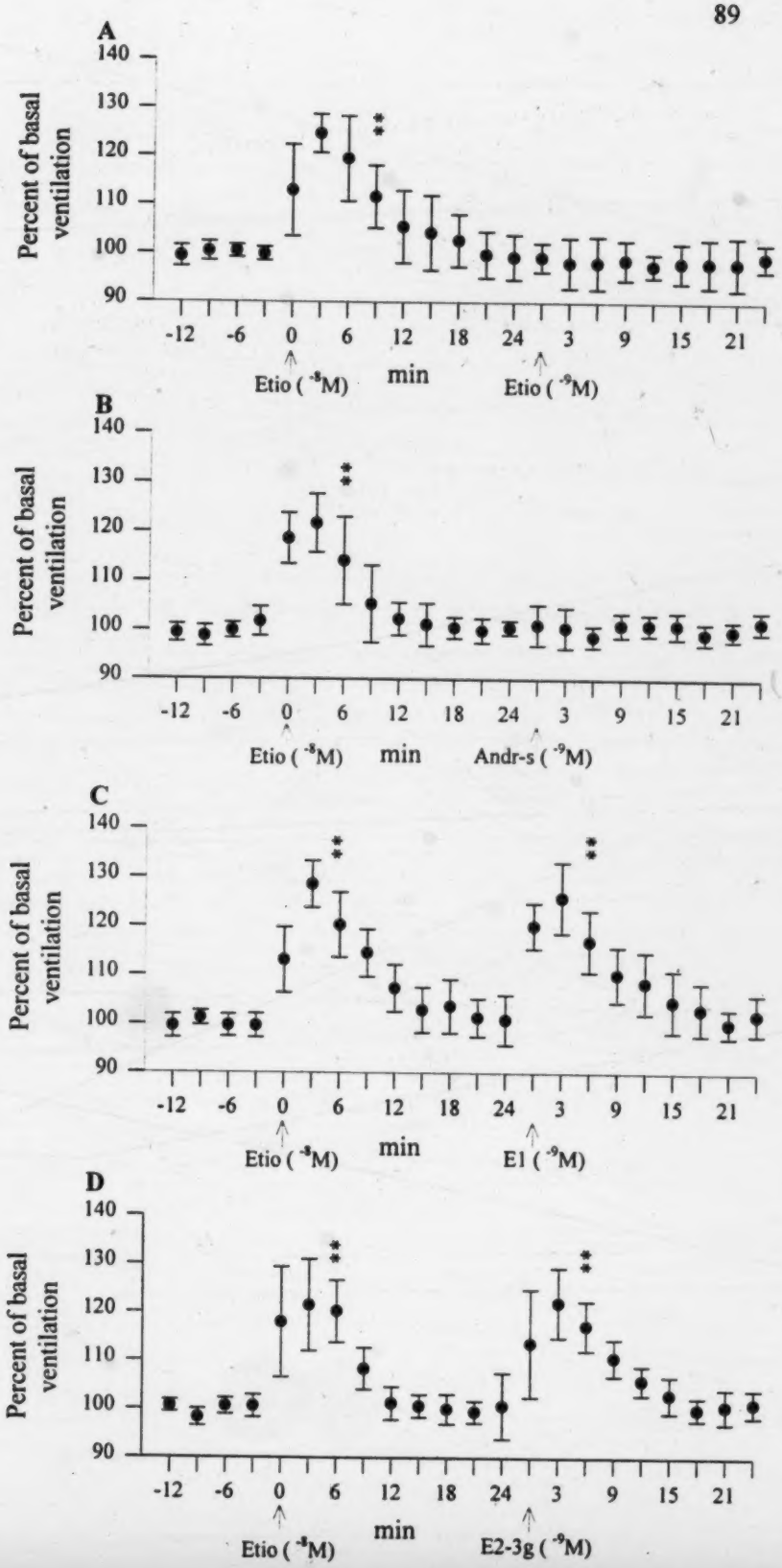

A
Percent of basal ventilation
140
130
120
110
100
90
-12 -6 0 6 12 18 24 3 9 15 21
min
Etio (-8 M)
Etio (-9 M)
B
Percent of basal ventilation
Etio (-8 M)
min
Andr-s (-9 M)
C
Percent of basal ventilation
Etio (-8 M)
min
E1 (-9 M)
D
Percent of basal ventilation
Etio (-8 M)
min
E2-3g (-9 M)

Figure 4.12. Percent change in basal ventilation rate (mean±s.d.) of males in behavioural cross-adaptation studies using 10^{-8} M DHEA-s as the adapting steroid. DHEA-s was added to the aquarium first, after a 12 min pretest (as indicated by the first arrow). After monitoring ventilation rates for 24 min, 10^{-9} M Andr-s was added to the aquarium and fish were observed for an additional 24 min. Ventilation rates in response to test substances were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding addition of first steroid). *N*=6. Statistical analysis of ventilation response was as described in Figure 4.2.

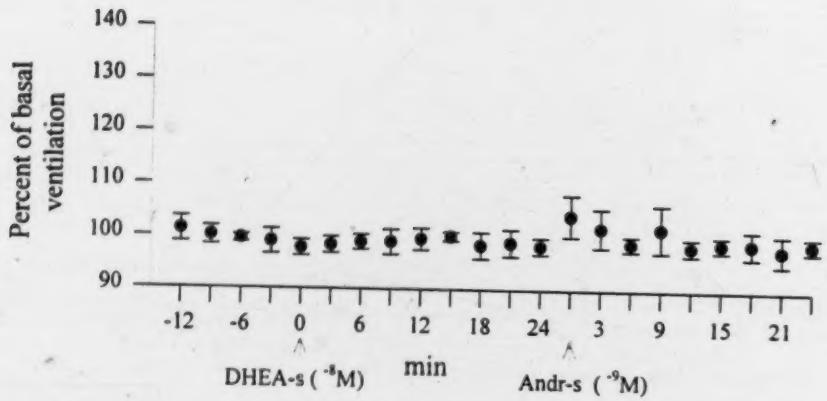
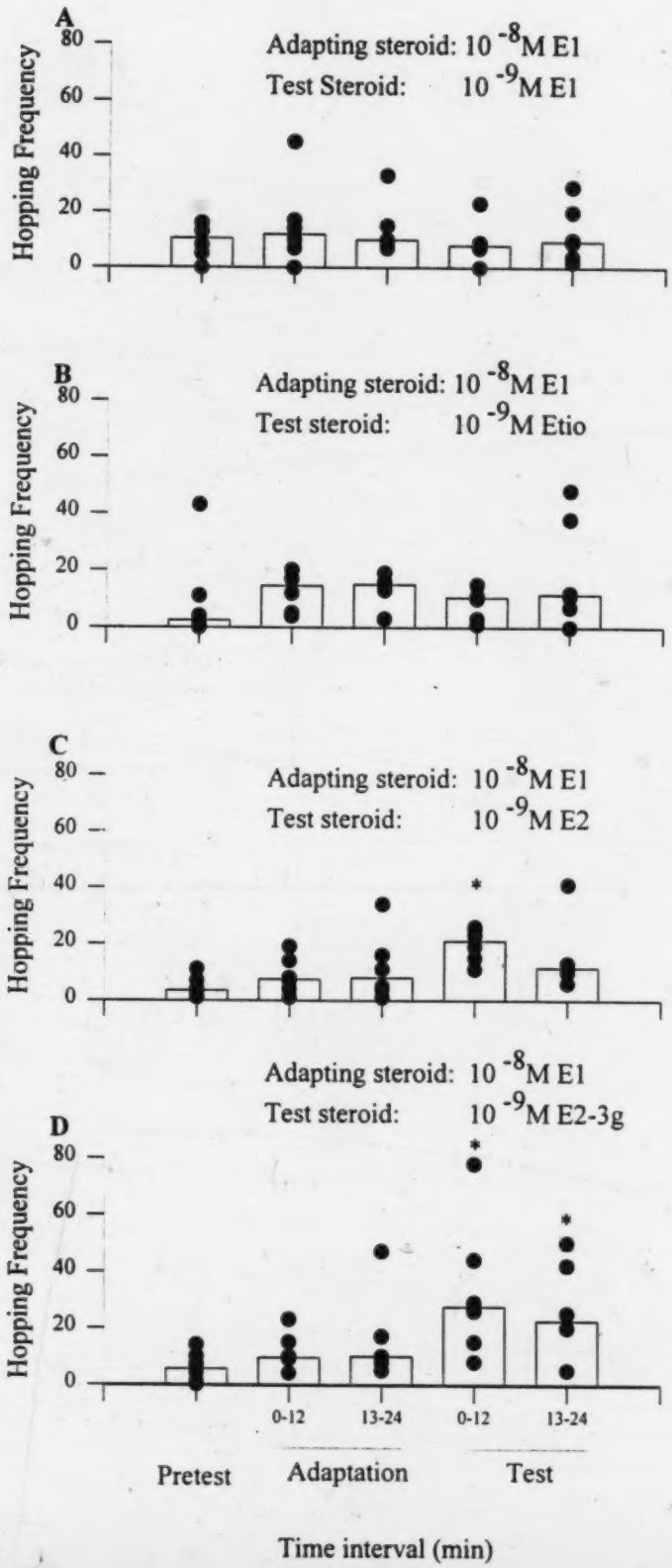

Percent of basal ventilation
140
130
120
110
100
90
-12
-6
0
6
12
18
24
3
9
15
21
min
DHEA-s (-8M)
Andr-s (-9M)

Figure 4.13. Changes in hopping frequency in males in behavioural cross-adaptation studies using 10^{-8} M E1 as the adapting steroid. In all cases, E1 was added to the aquarium first, after a 12 min pretest. After monitoring hopping frequencies for 24 min, a second steroid (A - 10^{-9} M E1; B - 10^{-9} M Etio; C- 10^{-9} M E2; D- 10^{-9} M E2-3g)was added to the aquarium and fish were observed for an additional 24 min. Hopping frequency during the 12 min pretest is compared to the first 12 min following addition of a test compound, and the next 12 min time interval. Bars represent median values. N=6 males in all cases. * median hopping frequency is significantly different than hopping frequency observed in the pretest (P<0.05).


A
Adapting steroid: 10 -8M E1
Test Steroid: 10 -9M E1
Hopping Frequency
80
60
40
20
0
B
Adapting steroid: 10 -8M E1
Test steroid: 10 -9M Etio
Hopping Frequency
C
Adapting steroid: 10 -8M E1
Test steroid: 10 -9M E2
Hopping Frequency
D
Adapting steroid: 10 -8M E1
Test steroid: 10 -9M E2-3g
Hopping Frequency
0-12
13-24
0-12
13-24
Pretest
Adaptation
Test
Time interval (min)

5. BEHAVIOURAL MASCULINIZATION OF FEMALES WITH METHYLTESTOSTERONE (MT)

5.1. METHODS

To determine if androgenic steroid hormones are responsible for male behavioural responsiveness to E1 and E2-3g, females were implanted with methyltestosterone (MT) (Sigma, St. Louis MO). Implants (12 mm length) were made from Dow Corning silastic tubing (1.98 mm i.d.; 3.18 mm o.d.) and had a bevel (2 mm) to facilitate insertion through a small abdominal incision. Both ends of the implants were sealed with 2 mm of silicon. (Sealastic; Dow Corning). The remaining 8 mm was either filled with approximately 3 mg of crystalline MT, or left empty (Blank implant). The bevel end of the implant was inserted through a small incision (<6 mm) made just anterior to the anus, and to the right of the midline of anesthetized (0.05% 2-PE) fish. Five females were implanted with MT and five with Blanks. Each fish was placed in a 70 L flow-through aquarium, provided with a single PVC tube and left to recover for one week. The aquaria were opaque on all sides, except for a small clear rectangle (length x width: 30 mm x 5 mm) on one end for viewing.

All females were tested twice, 8-10 days, and 16-18 days after implantation, using a similar protocol to that described in Section 4.1.2. After recording ventilation during a 12 min pretest, 10 ml of 1% ethanol (control) were added to the test aquaria, and the fish were observed for an additional 15 min. Three steroids (10^{-9} M) were added in sequence, with 15 min between each addition The first two steroids, E1 and E2-3g, act via separate

receptor classes and elicit behaviour changes only in males (see section 4.2.1, 4.2.3). The third steroid (Etio) acts through a third receptor class and increases ventilation rate in both males and females (see section 4.2.1). Etio was included in this experiment not to examine the effect of MT implant, but to ensure that females were capable of exhibiting a ventilation increase.

Four weeks after implantation, four females from both the MT and blank implant group were placed in 0.05% 2-phenoxyethanol for half an hour and any colour change was noted.

Ventilation rate data were analyzed as in section 4.1.2.4. As well, because ventilation responses to three test steroids were being considered, all three means of the ventilation rates recorded for the 4th and 7th min after a steroid addition were compared to the pretest mean using Student T-Tests with a Bonferroni adjustment ($p=0.05/3$) (Systat, 1994). Additionally, significance of differences between ventilation responses 8-10 days and 16-18 days after implantation was determined for each of the three steroids by one-tailed Wilcoxon paired test for non-parametric data (Systat, 1994).

Other behaviour occurrences were again summed for the 12 min pretest and also for each 12 min period following exposure to a test solution. The data were then compared using a Kruskal Wallis ANOVA for non parametric data (Systat, 1994).

5.2. RESULTS

As in previous experiments (Section 4.2.1), addition of 10 ml ethanol did not affect ventilation rate in females tested 8-10 days or 16-18 days after implantation with MT or Blank capsules (Fig. 5.1). Exposure to 10^{-9} M E1 or E2-3g did not increase ventilation rate of Blank-implanted females either 8-10 or 16-18 days after implant, whereas exposure to Etio induced an increase of similar magnitude in both tests (Fig. 5.1). MT-implanted females were similar to Blank females in exhibiting equivalent ventilation responses to Etio on the two tests. However, unlike Blank females, MT-implanted females significantly increased ventilation in response to either E1 or E2-3g, and these responses were significantly greater in the second test (16-18 days after implant) than in the first (Fig. 5.1).

When implanted females were anesthetized in 0.05% 2-PE for 30 min, the four females with MT-implants developed the black colour typical of courting males, with the coloration change extending to fins. In contrast, the four females with blank-implants darkened only slightly while their fins remained a yellowish-green colour (Fig 5.2).

Figure 5.1. Percent change in basal ventilation rate (mean±s.d.) in females that had been implanted with: blank silastic implants and methyltestosterone (MT) filled silastic implants after addition of a series of test compounds. 100 µl of ethanol (EtOH) was added to the water after a 12 min pretest (indicated by first arrow). Fifteen min later, 10^{-9} M E1 was added, followed by 10^{-9} M E2-3g, and 10^{-9} M Etio; each steroid was added after a 15 min observation period. Females were observed: A- 8-10 days after implantation; B - 16-18 days after implantation. Ventilation rates in response to test compounds were calculated as a percentage of the basal ventilation rate (ie. the mean ventilation rate in the 12 min preceding additions). *N*=5 for all groups. *, mean ventilation rate measured for the 4th and 7th min after addition of a steroid is significantly different than mean pretest ventilation rate (Bonferroni adjusted $P<0.05/3$).

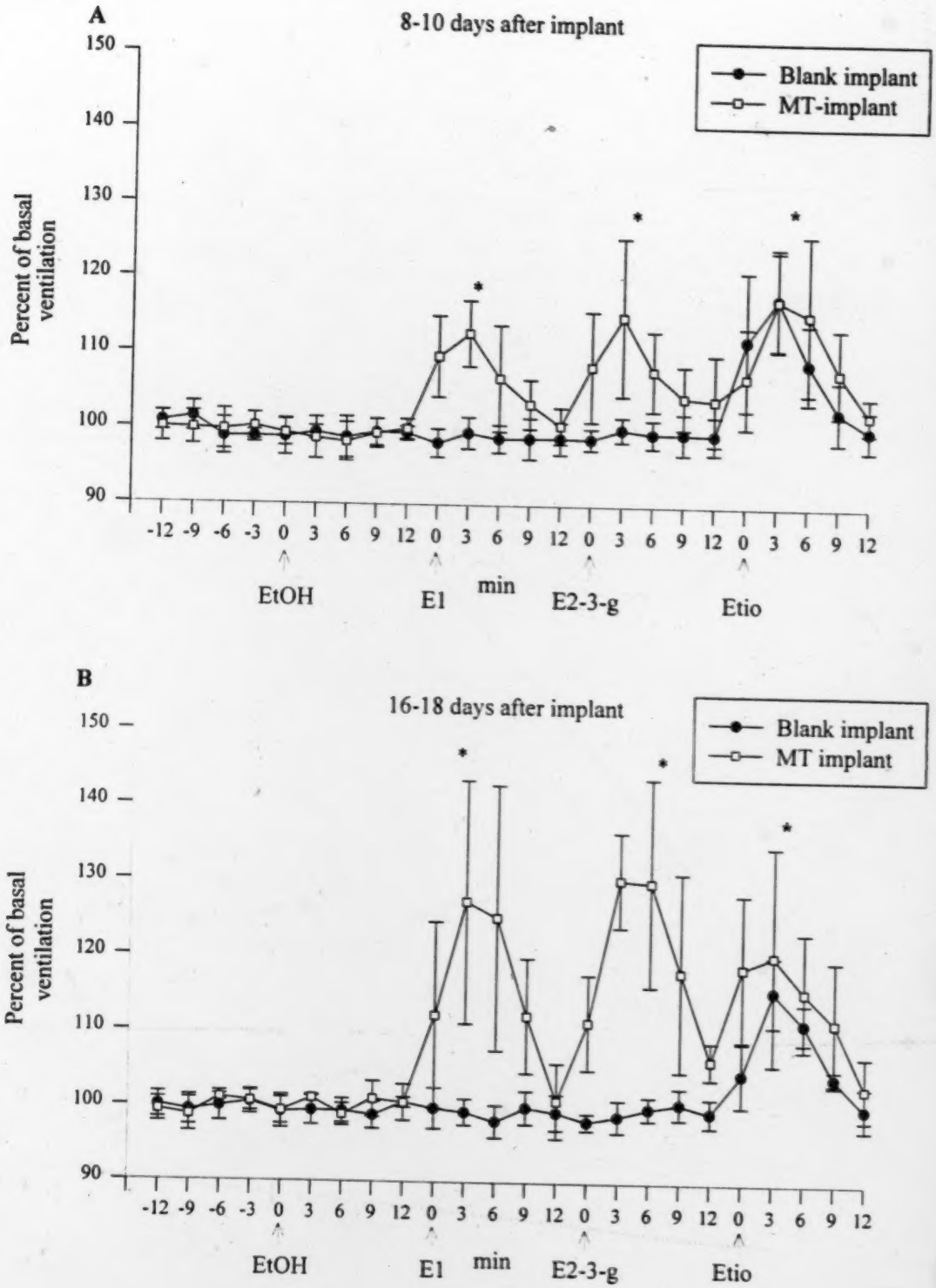

A
8-10 days after implant
Blank implant
MT-implant
Percent of basal ventilation
150
140
130
120
110
100
90
-12 -9 -6 -3 0 3 6 9 12 0 3 6 9 12 0 3 6 9 12 0 3 6 9 12
EtOH
E1
min
E2-3-g
Etio
B
16-18 days after implant
Blank implant
MT implant
Percent of basal ventilation
150
140
130
120
110
100
90
-12 -9 -6 -3 0 3 6 9 12 0 3 6 9 12 0 3 6 9 12 0 3 6 9 12
EtOH
E1
min
E2-3-g
Etio

Figure 5.2. Female *Neogobius melanostomus* after immersion in 0.05% 2-phenoxyethanol for 30 minutes. Thirty days prior to photograph being taken, the top female (black) was implanted with MT and the lower female was implanted with an empty capsule.

Manostat

6. DISCUSSION

This study shows that the olfactory epithelium of *Neogobius melanostomus* detects four classes of steroids, but does not detect prostaglandins. Detection of etiocholanolone-glucuronide (Etio-g) confirms previous biochemical and behavioural studies showing pheromonal function for Etio-g in another gobiid (*G. jozo*), and therefore suggests this steroid may commonly function as a pheromone in gobies (Colombo *et al.*, 1982). Results of this study are also similar to studies of hormonal pheromones in other perciforms, which demonstrate that prostaglandins are not detected (Stacey and Cardwell, 1995). The EOG results also indicate a lack of sexual dimorphism in detection of steroids, which is supported by other studies on teleosts such as the goldfish (Sorensen *et al* 1987; Sorensen *et al.*, 1995b) and the tinfoil barb (Cardwell *et al.*, 1995). Behavioural cross-adaptation studies using ventilation increase as a behavioural bioassay show that three of the four detected steroid classes are also perceived by *Neogobius*; unlike olfactory sensitivity, sexual dimorphism is evident in the behavioural response to detected steroids. Furthermore, it appears that the behavioural response of males to two steroid classes (E1 and E2-3g) is androgen-dependent.

Although failing to demonstrate reproductive response to the detected steroids, the combination of EOG results and the behavioural responses to detected steroid classes provide the most detailed data set on putative perciform pheromones, and suggests that *Neogobius* uses a complex chemical communication system. The results of this study are important because the order Perciformes is the largest order of vertebrates, and species

from this order dominate marine ecosystems (Nelson, 1994); pheromones used by this group are, for the most part, unknown.

6.1. Olfactory responses to steroids and steroid metabolites

6.1.1. Cross-adaptation

The olfactory organ of the round goby detects a wide variety of steroids, which were shown by EOG cross-adaptation studies to be detected by four classes of receptor mechanisms (estrone, estradiol-glucuronide, etiocholanolone and DHEA-sulphate) with at least two compounds appearing to operate via two receptor mechanisms (Tables 3.2 and 3.3). Receptor classes were named after the most potent steroid, as determined from EOG concentration-response studies. Although I have assumed that the most potent steroids are the most likely pheromonal candidates, it is possible that the most potent compound for each receptor class is not the actual pheromone, but a closely related compound. However, I tested most of the commercially available steroids that I felt, based on other studies, were likely pheromonal candidates. To determine which compounds is the natural ligand for each receptor class, one must determine what is released by the fish.

When using EOG recordings to identify olfactory stimulants, it is necessary to use cross-adaptation to determine olfactory discrimination, because if compounds are detected via separate olfactory receptor mechanisms, they may have separate functions. However, when interpreting cross-adaptation results, we make two assumptions that still need further investigation: 1) each receptor neuron expresses one receptor type, and 2)

each receptor type is designed to bind to one specific ligand, or compounds that approximate the structure of the ligand. The first assumption is based on work that showed individual receptor clones will anneal to a small percent (<2%) of olfactory neurons, suggesting that a single cell will only express a small subset of receptors (possibly one) (Ngai *et al.*, 1993). The second assumption arises from the identification of G-protein linked olfactory receptors with 7 transmembrane domains (Buck and Axel, 1991). Specificity of the olfactory receptor to particular ligands is proposed to be accomplished by slight alterations within a transmembrane domain (Buck and Axel, 1991).

Cross-adaptation is not without its problems. As first noted by Caprio and Byrd (1984), for amino acid assignment to specific olfactory transduction processes in the channel catfish (*Ictalurus punctatus*) grouping compounds into distinct receptor classes is difficult if olfactory receptors possess multiple binding sites. Using the terminology adopted by Shepherd (1987), an odour molecule is labelled an odogen, and the portion of the odogen that binds to one of the multiple binding sites within an olfactory receptor is called an odotope. It is possible that an olfactory receptor will show strong binding affinities to several different odogens that have one or more odotopes that bind to sites on an individual receptor (see Hildebrand and Shepherd, 1997 for review). Alternatively, an odogen that has one or more odotopes may have strong binding affinities for one or more olfactory receptors (this is discussed more fully below with respect to androsterone-sulphate [Andr-s]). Additionally, multiple binding sites within an olfactory receptor will confuse cross-adaptation results because an adapting odogen could bind to one site and

allosterically affect the binding properties of the other site(s), resulting in non-reciprocal cross-adaptation among compounds thought to share the same transduction mechanism (Caprio and Byrd, 1984).

Interpretation of cross-adaptation results also is confounded by my observation that repeated, long-interval (30 min) exposure to the same test compound (a control for cross-adaptation) reduced the magnitude of the EOG response to most test compounds without exposure to an adapting compound and, for some test compounds, eliminated EOG response to a second exposure (Fig. 3.5). This rapid degradation of EOG response has not been reported previously in fish EOG studies, and the mechanism behind the phenomenon is not clear. The degradation of the response indicates that even a brief exposure to an odogen can initiate long-term change in olfactory receptor function.

The order of steroid exposure in the EOG cross-adaptation experiments becomes important if exposure to one compound results in degradation of EOG responses of all compounds detected via the same receptor mechanism. For example, Etio-g and Etio, two compounds that share the same receptor mechanism when tested individually, induce EOG responses of similar magnitudes at 10^{-8} M (Fig. 3.3). However, when Etio is tested before Etio-g, the EOG response to Etio-g is much lower than the EOG response to Etio (Fig. 3.5). Maintaining a constant order of compound exposure for all cross-adaptation studies, and comparing the reduction of test compounds response magnitude to the reduction observed in the control, should separate receptor mechanisms.

Despite the problems inherent in the cross-adaptation procedure, the results of this EOG cross-adaptation study do suggest the existence of four receptor mechanisms in the

round goby, each with distinguishing characteristics.

6.1.2. Proposed classes of olfactory steroid receptors

6.1.2.1. Estrone (E1) receptor

The proposed E1 receptor responded to unconjugated C18 steroids: E1, E2 and E2α. The odotope recognized by the receptor is likely the 18 carbon backbone, since substituting an α hydroxyl for the β hydroxyl on C17 in E2α slightly shifts the first two carbon rings (A, B) into a different plane (see estradiol-17α in Appendix D - E1 receptor) and greatly reduces the EOG response (Table 3.1). Addition of another hydroxyl group on C16 (estriol; 1,3,5,[10]-estratrien-3α,16α,17β-triol) renders the odogen undetectable. Also, addition of a glucuronide at C3, as is the case with E2-3g, makes E2 unrecognizable to the E1 receptor, perhaps because it shifts the cyclopentane portion (D) into another plane. Steroids binding to the E1 receptor exhibit strong degradation of EOG response after initial exposure (Fig. 3.5).

The unconjugated C18 steroids used in the initial EOG screening appear to be detected by only a few species of non-perciform fish (in orders Cypriniformes and Characiformes) that have been screened for olfactory sensitivity to hormone and hormone metabolites using procedures described in this study (Stacey and Cardwell, 1995). While many species have not been tested, it appears as though this is the first report of detection of unconjugated estrogens in the Order Perciformes. Other tested Perciforms include: Eurasian ruffe, *Gymnocephalus cernuus* (Sorensen unpubl.); perch, *Perca flavescens* (Stacey *et al*, 1994); gourami, *Trichogaster trichopterus* (Stacey *et al* 1994); *Channa* sp.

(Stacey *et al*., 1994); cichlids, *Sarotherodon mossambicus, Symphysodon discus* (Stacey *et al*., 1994) and *Haplochromis burtoni* (Robison, unpubl.); and one species from the Family Scatophagidae (Stacey, unpubl.).

6.1.2.2. Estradiol-glucuronide (E2-3g) receptor

Three seemingly diverse steroid molecules (E2-3g, DHEA-g and Epiandr-g) appear to share the E2-3g receptor mechanism. The odotope for the proposed E2-3g receptor appears to be the glucuronide conjugate bound to C3, because the three effective odogens have the glucuronide moiety in approximately the same configuration with respect to the rest of the molecule, whereas other glucuronides that do not stimulate the receptor (Etio-g, or 3α,17α-5BP-3g) have the glucuronide in a different configuration (see Appendix D - E2-3g receptor). Both E2-3g and DHEA-g reduce the response to Epiandr-g, although only the effect of DHEA-g adaptation is significant (Fig. 3.7b). Epiandr-g needs to be tested in additional cross-adaptation experiments, as it may also share another receptor mechanism (Etio). Compounds that are adapted by E2-3g (particularly DHEA-g) show a strong degradation of EOG response after a second exposure (Fig. 3.5).

C18 conjugates are reported to have pheromonal function in other fish species. E2-3g has been reported to attract male zebrafish, *Danio rerio* (van den Hurk and Lambert, 1983). However, E2-3g does not induce EOG response in zebrafish (Stacey and Cardwell, 1995). As well, studies using voltage sensitive dyes applied to the olfactory bulb of the zebrafish show that E2-3g does not cause fluorescence in glomeruli; other compounds that induce EOG responses in zebrafish (amino acids, 17,20βP-20s, PGs) will induce fluorescence in subsets of glomeruli (Korsching, 1997). E2-3g, a C18 with both a

glucuronide and sulphate (1,3,5,[10]-estratrien-3-sulphate-17-glucuronide) and a number of other C18 sulphates induce EOG responses in another perciform, the African cichlid (*H. burtoni)* (Robison, unpubl. data). Estradiol-sulphates, such as E2-3s (1,3,5[10]-estratriene,3α,17β-diol-3-SO4) induce EOG responses in a number of species from the order Characiformes (Cardwell and Stacey, 1995a).

6.1.2.3. Dehydroepiandrosterone-sulphate (DHEA-s) receptor

Similar to the E2-3g receptor, the conjugate appears to be the odotope for the proposed DHEA-s receptor mechanism (see Appendix D - DHEA-s receptor). Unconjugated DHEA does not elicit an EOG response, whereas DHEA with a sulphate located on C3 does. DHEA-s also adapts the EOG response to Andr-s, another compound with a sulphate positioned on C3. Andr-s also appears to interact with the Etio receptor (Fig 3.8).

DHEA-s also adapts 3α,17α-5βP-3g, another steroid that acts via the Etio receptor (Fig. 3.8). It is not surprising that 3α,17α-5βP-3g is adapted by Etio, as its unconjugated form is completely adapted by Etio, and the main carbon rings are likely to be recognized by the generalist Etio receptor. What seems unusual is that a glucuronated steroid will also bind to an apparent sulphate receptor mechanism (DHEA-s). The receptor may recognize a conjugate on C3, but this would not explain why DHEA-s does not adapt other glucuronides like Etio-g. The explanation for this phenomenon remains to be determined. Compounds sharing the DHEA-s receptor mechanism do not show degradation of EOG response on second exposure (Fig. 3.5).

DHEA-s is known to induce EOG response in only one other species, the cichlid

H. burtoni (Robison, unpubl. results).

6.1.2.4. Etiocholanolone (Etio) receptor

The proposed Etio receptor seems to be relatively non-specific, and the odotope is likely the main carbon chain (see Appendix D - Etio receptor). The receptor may recognize the structure formed from C1 through C10 (A, B), as this conformation of the molecule seems to be conserved in all the seemingly diverse free and conjugated C19 and C21 compounds detected via this mechanism (Tables 3.2 and 3.3). Two compounds interacting with the Etio receptor, Andr-s and 3α,17α-5βP-3g, also interact with the DHEA-s receptor (see Appendix D - DHEA-s receptor) The Etio receptor likely recognizes the main carbon chain as the odotope, whereas the DHEA-s receptor recognizes the conjugate. Although not examined in this series of cross-adaptation experiments, Epiandr-g may also have affinity for the Etio receptor. Compounds detected via the Etio receptor show degradation of EOG response on second exposure (Fig. 3.5).

Detection of Etio-g by the round goby is a significant finding consistent with earlier work (Colombo *et al.*, 1982) suggesting that this compound is released by male *G. jozo* to attract females and stimulate oviposition. Interestingly, all of the detected 5β-reduced androgens, C21 compounds and AD, compounds proposed to be byproducts of the hydroxylation of pregnenolone in the Leydig cell-rich mesorchial gland in the black goby (Colombo *et al.*, 1977) are detected via the same receptor mechanism. Additionally, Etio, 3α,17α-5βP, 3α,17α-5βP-3g, and 11β-Etio-g, steroids reported to be produced in the seminal vesicles (an area that also has a high concentration of Leydig cells) of the African catfish (Resink *et al.*, 1989a; Schoonen *et al.*, 1988) induce EOG

responses in the round goby. These three compounds may function to attract female *Neogobius*, and the latter two are also potent olfactory stimulants in the African catfish (Resink *et al.*, 1989b).

6.1.3. Olfactory insensitivity to prostaglandins

None of the prostaglandins tested in this study induced EOG responses in the round goby. Although the majority of species tested, using the EOG procedure, show olfactory sensitivity to PGs (see Stacey and Cardwell, 1995 for review), most of these species are from the Orders Cypriniformes, Characiformes and Salmoniformes, in which all species tested respond to PGs. The present findings in the round goby are consistent with studies on other perciforms, none of which detect PGs (see Stacey *et al.*, 1994 and Stacey and Cardwell, 1995 for reviews; *Haplochromis burtoni* (Robison, unpubl. data); Eurasian ruffe, (Sorensen, unpubl. data)). PGs also do not induce EOG responses in the few tested representatives from the Orders Acipenseriformes, Osteoglossiformes and Gadiformes (Stacey and Cardwell, 1995). Although the function of PGs as potent post-ovulatory pheromones is confirmed or suspected in just a few fish species, such as the goldfish (Stacey, 1981, 1987; Sorensen *et al.*, 1986, 1988, 1989, 1995a), Atlantic salmon (Moore and Waring, 1995; Waring and Moore, 1995), and the loach (Kitamura *et al.*, 1994a; Ogata *et al.*, 1994), this and previous EOG studies indicate the use of PGs as pheromones is not universal.

When goldfish are injected with PGF2α, a number of unidentified PG metabolites are released (Sorensen *et al.*, 1995a), indicating that there likely are many

unidentified PGs that could have pheromonal function in fish. Thus, it is possible that the goby detects an unidentified prostaglandin or prostaglandin metabolite not tested in this EOG study.

6.1.4. Olfactory insensitivity to maturation inducing steroids (MIS)

Although the round goby detected several C21 compounds, steroids that typically induce maturation of oocytes in other teleosts did not induce EOG response. Two MIS such as 17,20β-P and 4-pregnen-17α,20β,21-triol-3-one (17,20β,21-P) have been shown to induce oocyte maturation in most fish tested (see Nagahama, 1994 for review), the latter steroid likely being the MIS in perciform species (Trant *et al.*, 1986; Trant and Thomas, 1989). 17,20β-P and metabolites function as potent preovulatory pheromones in several cyprinid species such as the goldfish (Stacey, 1987; Stacey *et al.*, 1989; Scott and Sorensen, 1994; Scott and Vermiessen, 1994; Sorensen and Scott, 1994), common carp (Irvine and Sorensen, 1993; Stacey *et al.*, 1994), and crucian carp (Bjerselius *et al.*, 1995b). Studies on another perciform, the Eurasian ruffe, indicate that males respond to a potent unidentified metabolite of 17,20β,21P (Murphy and Sorensen, unpubl. data). Therefore, it is possible that the round goby is sensitive to an unidentified MIS metabolite that was not tested in this study. It also is possible that MIS are not important chemical cues in round goby reproductive communication.

6.2. Behaviour

None of the four proposed steroid classes induced any reproductive behaviour. There are four main reasons why this might happen. First although the detected steroids might normally induce reproductive behaviours, responses might be exhibited only when the steroids are detected together with additional cues, which were absent in the present experiments involving isolated fish. Second, gobies may use mixtures of steroids for reproductive cues, instead of the single compounds that were tested in this study. Third, the detected steroids may have functions other than eliciting a behavioural response. Fourth, fish kept in laboratory conditions may not have been in the proper physiological state to respond to sex pheromones.

6.2.1. Additional cues

Classic studies by Tavolga (1956) first established that one gobiid species, *Bathygobius soporator*, uses both chemical cues and non-chemical cues (acoustical and visual) in reproductive signalling. Male *B. soporator* produce low-pitched grunts when courting females (Tavolga, 1958). Acoustic signals have been recorded from *Neogobius* (Protosov *et al.*, 1965) and other species, such as the common goby, *Padogobius martensii*, and the panzarolo goby, *Knipowitschia punctatissima*, also emit trains of low frequency pulses when courting or spawning (Lugli *et al.*, 1995). Additional support for the use of acoustic signals by *Neogobius* are my observations that gaping and coughing behaviours were associated with the production of non-harmonic sounds (Appendix B and C). Although the function of gaping and coughing is not clear, both behaviours may

. be social vocalizations used to signal conspecifics, as field observations show that the round goby tends to aggregate in large social groups during the spawning season (Murphy, personal observation).

Visual cues, such as behavioural displays and colour changes, are common in gobiid spawning habits (Breder and Rosen, 1966). The extreme colour change exhibited by male *Neogobius* when kept in an aquarium containing females suggests that the round goby also uses visual displays during courtship. The apparent use of multiple communication systems by the round goby suggests an elaborate courtship and spawning display; thus, it is possible that a single chemical signal without accompanying visual or acoustical cues may be out of reproductive context for the goby. In contrast, feeding behaviour is readily induced with chemical signals alone (Fig. 4.6).

The inability of single detected steroids to elicit reproductive behaviours also might be due to the absence of additional chemical cues which could be hormonal or non-hormonal. The possibility of hormonal mixtures is considered in the following section.

6.2.2. Chemical mixtures

Tavolga (1956) was able to induce courtship behaviour in male *B. soporator* using only chemical cues (ovarian fluid from ovulated females) as a stimulus. Ovarian fluid likely contains more than one type of detected pheromone. It is also possible that urine can induce reproductive behaviour in the round goby, as female *G. jozo* are attracted to male urine (Colombo *et al.*, 1982). Urine of the round goby may also contain a variety of steroids, as it does in the Pacific herring, *Clupea harengus*, and North Sea plaice,

Pleruonectes platessa (Scott *et al.*, 1991). It is possible that a single compound is insufficient to induce reproductive behaviour in the round goby, and that gobies rely on mixtures in which the ratios of active components are important. Mixtures of chemicals in species-specific ratios are critical for inducing behavioural responses in insects (see Kaissling, 1996 for review).

Ovulated female *G. jozo* are attracted to single steroids (Colombo, *et al.*, 1982) and single compounds are also effective pheromones in goldfish and carp (e.g. PG and 17,20β-P) (see Stacey *et al.*, 1994 for review). Female *Neogobius* may not have responded to single steroids because of their reproductive condition. Male *Neogobius* may require chemical mixtures, rather than single compounds, as a reproductive cue.

An unexpected result of treating female gobies with MT (Chapter 5) may provide insight into the use of chemical mixtures by the round goby. Although males were observed to develop dark colouration only when kept in an aquarium with females, solitary females that have been implanted with MT occasionally develop the dark colouration typical of a courting male (Murphy, personal observation). Assuming that the dark colouration of males is induced by the odour of females, it is possible that androgen-implanted females respond to their own odour. As studies on androgen-implanted female goldfish show that androgen treatments do not disrupt vitellogenesis or ovulatory GtH-II surges (Kobayashi *et al.*, 1989; Kobayashi and Stacey, 1990; Stacey and Kobayashi, 1996), it is possible that MT implants in female gobies did not alter their production and release of gonadal steroids. Rendering a solitary MT-implanted female anosmic should indicate if the female changes colour in response to her own odour. Single steroids failed

to induce a colour change in males, suggesting that a pheromone component is missing.

It also is possible that gobies respond to a temporal sequence of odours. For example, an increase in hopping behaviour (a behaviour that appeared to be a search behaviour), was observed in the behavioural cross-adaptation experiment when E2 and E2-3g were adapted by E1 (Fig.4.13c, d) . However, a mixture of all the compounds added to the aquarium did not induce this same pattern of response. The increase in hopping behaviour was exhibited only after a previous exposure to E1, raising the possibility that the goby responds to sequential pulses of different steroids. Recent studies on goldfish suggest that fish may use temporal patterns of pheromonal prostaglandin release to communicate reproductive status (Appelt and Sorensen, unpubl.)

Although single steroids failed to induce reproductive behaviour, single steroids consistently induced a ventilation rate response. This suggests that single steroids are sufficient stimuli to generate afferent activity that reaches central nervous system sites regulating the motor patterns of ventilation and raises questions about how mixtures of steroids are encoded. If reproductive behaviour is only achieved through exposure to mixtures of steroids, rather than single compounds, it is possible that neuronal pathways that mediate the response to mixtures differ from those that induce a ventilation response, and that reproductive behaviour may be activated only after integration of bulbar inputs generated by exposure to steroid mixtures.

6.2.3. Pheromones with priming effects

Absence of a behavioural response to detected steroids might be expected if the

steroids induce physiological (priming) effects rather than behavioural (releaser) effects (Wilson and Bossert, 1963). As in goldfish (Dulka *et al.*, 1987; Stacey *et al.*, 1989). and common carp (Stacey *et al.*, 1994), the steroids detected by *Neogobius* may induce physiological changes in preparation for spawning, such as increases in GtH levels, milt volumes and circulating steroid levels. This study did not attempt to measure any physiological changes.

6.2.4. Inappropriate reproductive condition

Although all fish used for behavioural experiments were sexually mature, some or all may not have been in the proper reproductive condition to respond. Only ovulated female *G. jozo* exhibit reproductive behaviour (attraction and oviposition) in response to Etio-g (Colombo, 1982). Additionally, the photoperiod and water temperature under which the fish were held may not have been suitable. However, during the course of the experiments, mixed sex pairs held under the standard photoperiod and temperature conditions did occasionally spawn and produce clutches. Therefore, considering the number of fish used for the behavioural studies, it is likely that at least some should have been in appropriate reproductive condition.

6.2.5. Ventilation behaviour

Three of the four steroid classes that evoked an EOG response in *Neogobius* also increased ventilation rates As well, the ventilation response was sexually dimorphic. Males increased ventilation frequency in response to steroids interacting with the

proposed Etio, E1 and E2-3g receptor mechanisms, whereas females increased ventilation rates only in response to steroids acting on the Etio receptor. The ventilation rate increase is not restricted to putative pheromonal stimuli, because exposure to food odour also increased ventilation.

An increase in the ventilation frequency might function to sample or "sniff" the surrounding water. In benthic fish, such as the flounders *Lepidopsetta bilineata* and *Platichthys stellatus*, water flow through the naris is achieved by a pumping mechanism in which changes in the volume of the nasal sac occur in synchrony with ventilation (Nevitt, 1991). Pipping (1927) also demonstrated that the black goby, *Gobius niger*, maintains a current of water through the olfactory chambers by using respiratory and ciliary movement, and is thereby able to perceive odours in stagnant water. The odor-induced ventilation increase characterized for the round goby in this study could serve as a useful behavioural bioassay for future studies of odour perception in benthic fishes.

Various characteristics of the increase in ventilation rate made it possible to explore two separate avenues of research. First, ventilation rate increase was used to confirm EOG findings concerning sensitivity to detected steroids and the number of steroid receptor mechanisms present. Second, the sexual dimorphism of the ventilation response (which was not evident in the EOG response), enabled me to determine if the dimorphism was androgen-dependent, and to consider how the dimorphism might be related to pheromonal function.

6.2.6. Relationship between EOG and behavioural responses to odours

Threshold steroid concentrations for the ventilation responses (Fig. 4.7) were one order of magnitude lower than threshold concentrations for EOG responses (Fig. 3.3): e.g. steroids such as E1, that have EOG threshold concentration at 10^{-9} M, have a behavioural threshold at 10^{-10} M. The olfactory threshold reported is probably a conservative estimate; in cases where mean responses to 10^{-10} M steroids were not significantly different from responses to background water, it often was clear that individual fish detected the steroids at this concentration (Fig. 3.3). Use of larger sample sizes likely would have lowered apparent olfactory detection thresholds in EOG recordings.

Another reason why sensory and behavioural thresholds differed may be the different protocols used for EOG and behavioural concentration response studies. In EOG recordings, the naris is exposed to known concentrations of test compounds, whereas in behavioural studies, the concentration of a test solution during dilution varies from test to test. When adding an odour to an observation aquarium, it is likely that the fish is initially exposed to a much higher concentration than anticipated, as it takes approximately 3 min for an odour to disperse. This phenomenon may explain why the threshold concentration for a ventilation response is lower than threshold for an EOG response; e.g. for Andr-s the threshold concentration for a ventilation response is 10^{-9} M (Fig. 4.9), whereas for an EOG response it is 10^{-8} M (Fig. 3.3)

Until now, fish studies using EOG cross-adaptation to determine the presence of multiple olfactory receptor mechanisms have focussed on sensory discrimination. The results of the behavioural cross-adaptation experiment clearly show that male *N.*

melanostomus can also behaviourally discriminate steroids detected by different receptor mechanisms (Figs. 4.10 and 4.11). Conversely, the data also suggest that steroids detected by the same receptor mechanism are not discriminated behaviourally.

Results from each behavioural cross-adaptation experiment are consistent with results from EOG cross-adaptation, with one unexpected finding. EOG cross-adaptation data suggest that Andr-s is detected by two of the receptor mechanisms, Etio and DHEA-s (Fig. 3.8). If Andr-s possesses odotopes that bind to DHEA-s and Etio receptors, then DHEA-s, a steroid that does not induce a ventilation response, should not adapt the ventilation rate response to Andr-s. Thus, during DHEA-s adaptation, Andr-s should be free to act via the Etio receptor. However, Andr-s did not significantly increase ventilation during DHEA-s adaptation (Fig. 4.12). This unexpected result might have been due to an ineffective Andr-s concentration (10^{-9} M), since EOG recordings indicate this concentration of Andr-s is not detected. Also the ventilation response to 10^{-9} M Andr-s (Fig. 4.9) appears smaller than that to 10^{-8} M Andr-s (Fig. 4.3), suggesting 10^{-9} M Andr-s is near the response threshold in the ventilation test. Thus, the apparent adaptation of 10^{-9} M Andr-s by 10^{-8} M Etio (Fig. 4.11b) should be viewed with caution, since the results could be due to insufficient Andr-s, rather than adaptation.

6.2.7. Possible pheromonal function of detected steroids

Although EOG responses are not sexually dimorphic, the sexual dimorphism of the ventilation rate response provides some insights into the possible pheromonal functions of the detected steroids.

6.2.7.1 C18 steroids (estrogens and their metabolites)

Only male *Neogobius* increase ventilation in response to C18 steroids. This ventilation response appears to be androgen-dependent because females, when implanted with MT, also increased ventilation rates in response to E1 and E2-3g. MT implant also induced in females the potential for display of dark colouration (Fig. 5.2), normally seen only in males. From these results, it is expected that juvenile male *Neogobius* would neither exhibit dark colouration, nor increase ventilation in response to water-borne C18 steroids. Androgen treatment would likely induce the potential for both behaviours. The mechanism(s) underlying the MT masculinization of the ventilation response and colour change are unknown. In the only other study of androgen-induced change in hormonal pheromone function (Cardwell *et al.*, 1995), androgen treatment of juvenile *Puntius* increased the EOG response to PGs and induced male sexual behaviour in response to PG-treated juveniles. The mechanism(s) of androgen action in *Puntius* differs from that in *Neogobius* because in *Puntius* the EOG response to PGs is sexually dimorphic, whereas in *Neogobius* the EOG response to C18 steroids is not (Fig. 3.2).

Because there is no evidence that C18 steroids are produced by male *G. jozo* (Colombo *et al.*, 1979), the detected C18 steroids are likely female pheromones that provide information to males. The C18 steroids also are detected via two separate receptor mechanisms, the E1 and E2-3g receptors, raising the possibility that C18 steroids might have more than one function. Studies on rainbow trout indicate that the route of pheromone release is dependent on steroid conjugation, conjugates being released in urine and feces, and free steroids likely being released across the gills (Scott and

Vermeirssen, 1994; Vermeirssen and Scott, 1995). If this is the case with *Neogobius*, it is possible that E2-3g, released in urinary pulses, is used to detect females at a distance, whereas E1, released tonically at low concentration, is used to identify the gender of females near or in the nest.

6.2.7.2. C19 and C21 steroids

Both males and females increased ventilation in response to compounds detected via the Etio receptor. Ventilation rate increases were observed in response to Etio-g (Fig.4.3). a compound previously reported to be released by male *G. jozo* to attract females and stimulate oviposition (Colombo *et al.*, 1982). Other compounds that are proposed to be metabolites of pregnenolone in the Leydig cell rich mesorchial gland in male *G. jozo*, AD and Etio, are detected by the same receptor mechanism, and presumably induce the same ventilation rate. Other compounds, such as 3α,17α-5βP, 3α,17α-5βP-3g, and 11β-Etio-g, produced in seminal vesicles of the African catfish (Resink *et al.*, 1989a; Schoonen *et al.*, 1988), are also detected by the Etio receptor and are expected to increase ventilation rate, although this was not examined. The general Etio receptor likely recognizes most of the compounds produced by Leydig cells..

The possibility exists that ligands detected by the Etio receptor are female pheromones, because females of other fish species have elevated circulating androgens, such as AD, during the spawning season (see Borg, 1994 for review). AD is proposed to function as a female pheromone in goldfish (Scott and Sorensen, 1994; Sorensen and Scott, 1994), and AD is detected by the Etio receptor mechanism of *Neogobius*. Defining the natural ligand for the Etio receptor mechanism will only be accomplished by

determining what hormones are released by male and female gobies. Because Etio-g has been identified as a pheromone in *G. jozo* (Colombo *et al.*, 1982), it is a pheromonal candidate in *Neogobius*.

6.2.7.3. DHEA-s

DHEA-s did not induce a ventilation rate increase in either gender. Because ventilation rate increases may function to prolong exposure to an odour, this steroid may not have pheromonal function. In *Neogobius*, there may not be an actual DHEA-s receptor mechanism, but rather another unknown steroid-sulphate receptor mechanism, in which DHEA-s shares only a small portion of the transduction mechanism. DHEA-s may be an inappropriate ligand to initiate a transduction mechanism and induce a behavioural response, or may require a higher concentration to induce a response.

6.2.7.4. Steroidal sex pheromones in the round goby

The results of this study suggest the round goby has evolved a complex sex pheromone system using a number of released steroids and steroid metabolites. There seems to be a female component, as well as a male. Since reproductive behaviour was not induced, one can only speculate on the function of these detected steroid classes.

The hormonal compounds that are released by females and that may have pheromonal function in the round goby appear to differ from those used by fish that employ a scramble competition type mating system in which males compete directly for access to ovulated females (Emlin and Oring, 1977). For example, the round goby does not detect the PGs commonly detected by many fish species (Stacey and Cardwell, 1995 for review), indicating that PGs may not have pheromonal function in this species.

Because in *G. jozo* only ovulated females are attracted to Etio-g (Colombo *et al.*, 1982), it is likely that only ovulated female *Neogobius* approach territorial males, a situation in which there may have been no selective pressure to evolve mechanisms for discriminating between ovulated and nonovulated females. Similarly, the apparent absence of olfactory response to maturation-inducing steroids likely results because males are not exposed to females during final oocyte maturation, but only after ovulation has occurred.

The round goby is not monogamous, and males will attract several females to a nest site and guard eggs oviposited from several females (Miller, 1984; Jude *et al.*, 1992). Since female egg production seems to be regulated by temperature and photoperiod (Moiseyeva and Rudenko, 1979), and females are multiple spawners, males may synchronize their territoriality and nest-building with female reproductive development by detecting estrogens produced and released during vitellogenesis (Specker and Sullivan, 1994). During ovarian development, estrogenic compounds (E1 and E2) produced by the ovary to induce vitellogenin production in the liver (see Nagahama, 1994 for review), may be released in free and conjugated forms (E2-3g), to function as pheromones inducing nest-building and courtship.

Once males initiate courtship, the male may switch roles from a pheromone receiver to a pheromone sender. From what is known about male teleost reproductive chemical signalling systems, and what the round goby detects, the chemical compound(s) that may be released by male *Neogobius* for the purpose of attracting females to nest sites, are likely a number of free and conjugated 5β-reduced androgens. Since Etio-g has been identified in *G. jozo* as a potential pheromone that attracts females and stimulates

oviposition. Etio-g (or a structurally similar compound like Etio) is also likely to have pheromonal function in *Neogobius*. The pheromone may be released via the urine, (Colombo *et al*., 1982), or by milt discharge on egg-laying substrates in preparation for female oviposition (Tavolga, 1954; Ota *et al*., 1996). It is possible that females respond to Etio to find a suitable nest site for oviposition, and a spawning male. Males may respond to Etio to monitor the proximity of other male's nest sites.

As in other gobiids (Magnhagen, 1989; Forsgren, 1997), male *Neogobius* may possess alternate mating strategies, with Type I males guarding nests and exhibiting paternal care, and Type II males showing sneaker traits. This speculation is based on my laboratory observations and observations from the field (MacInnis, personal communication) that two male morphs exist. Type I males have gonadosomatic indices (GSI) of approximately 2, are larger (> 80 mm) and possess male secondary traits such as enlarged cheeks. In contrast, Type II males, are similar in appearance to ovulated females, have proportionately larger testes (GSI of approximately 4), are typically smaller in length (< 70 mm), have greatly extended urogenital papilla and reduced mesorchial glands. If two morphs exist, sneaker males may respond to Etio to find a potential Type I male's nest site.

During courtship and spawning at the nest site, males may respond to E1, expected to be released continuously by vitellogenic females. The E1 pheromone may be particularly important within a dark nest, where visual cues might be ineffective. Also, if male *Neogobius* do exhibit two mating strategies, E1 pheromone may enable Type I males to discriminate females from Type II males.

6.3. Summary

This study demonstrates that *Neogobius* detects and responds to a variety of water-borne steroids and steroid metabolites. Although the findings suggest that *Neogobius* has evolved a complex sex pheromone system, further research is required to determine the putative pheromonal functions of the detected steroids. Such research is warranted for two reasons. First, the goby pheromone system is the first to be explored in a perciform fish, and may serve as a new model system for paternal nest-guarding species. Second, further understanding of the round goby pheromonal system may indicate feasible approaches to control the spread of this species in the Great Lakes and other North American aquatic ecosystems.

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APPENDIX A . Steroids and prostaglandins tested in EOG

STEROIDS:

Supplier	Cat #	Chemical name
		5a-androstan free (19 C)
Steraloids	A 1630	5a-androstan-3,17-dione
Steraloids	A 2490	5a-androstan-3B-ol-17-one
Sigma	A 8380	5a-androstan-17B-ol-3-one
		5a-androstan-SO4 (19 C)
Steraloids	A 2460	5a-androstan-3a-ol-17-one-SO4
		5B-androstan (19 C)
Steraloids	A 3270	5B-androstan-3,17-dione
Steraloids	A 3670	5B-androstan-3B-ol-17-one
Sigma	E 5126	5B-androstan-3a-ol-17-one
		5B-androstan-gluc
Lambert		5B-androstan-3a,17B-diol-17B-gluc
Lambert		5B-androstan-3a,17B-diol-3a-gluc
Steraloids	A 3626	5B-androstan-3a-ol-17-one-3-gluc
		4-androsten free (19 C)
Sigma	A 9630	4-androstene-3,17-dione
Steraloids	A 6630	4-androsten-11B-ol-3,17-dione
Sigma	T 1500	4-androsten-17B-ol-3-one
Sigma	H 4128	4-androsten-11B,17B-diol-3-one
Sigma	K 8250	4-androsten-11-keto-17B-ol-3-one
Sigma	E 5878	4-androsten-17a-ol-3-one
Steraloids	A 6600	4-androsten-11a-ol-3,17-dione
		4-androsten-11-keto-3,17-dione
Sigma	N7252	4-estren-17B-ol-3-one
		4-androsten-gluc
Sigma	T 2000	4-androsten-17B-ol-3-one-17B-gluc
		4-androsten-SO4
Steraloids	A 7010	4-androsten-17B-ol-3-one-17B-SO4
		5-androsten free
Steraloids	A 8500	5-androsten-3B-ol-17-one
Sigma	A 8755	5-androsten-3B,17B-diol
		5-androsten-gluc
Steraloids	A 8515	5-androsten-3B-ol-17-one-3-gluc
		5-androsten-SO4
Steraloids	A 8530	5-androsten-3B-ol-17-one-3-SO4

Supplier	Cat #	Chemical name
		estrogens free (18 C)
Sigma	E 9750	1,3,5(10)-estratrien-3a-ol-17-one
Sigma	E 8875	1,3,5(10)-estratrien-3a,17B-diol
Sigma	E 1253	1,3,5(10)-estratrien-3a,16a,17B-triol
Steraloids	E 870	1,3,5(10)-estratrien-3a,17a-diol
		estrogens-gluc
Sigma	E 2127	1,3,5(10)-estratrien-3a,17B-diol-3-gluc
Sigma	E 1127	1,3,5(10)-estratrien-3a,17B-diol-17-gluc
		estrogens SO4
Sigma	E 9505	1,3,5(10)-estratrien-3a,17B-diol-3-SO4
Steraloids	E 1103	1,3,5(10)-estratrien-3a,17B-diol-17-SO4
Steraloids	E 1050	1,3,5(10)-estratrien-3a,17B-diol-di-SO4
Sigma	E 1636	1,3,5(10)-estratrien-3a,17B-diol-di-SO4
		estrogens mixed conjugates
Sigma	E 2128	1,3,5(10)-estratrien-3-gluc-17-SO4
Sigma	E 3890	1,3,5(10)-estratrien-3-SO4-17-gluc
		5a-pregnan free (21 C)
Steraloids	P 2750	5a-pregnan-3,20-dione
Steraloids	P 3700	5a-pregnan-17-ol-3,20-dione
Steraloids	P 2320	5a-pregnan-17,21-diol-3,20-dione
Steraloids	P 5250	5a-pregnan-11B,17,21-triol-3,20-dione
Steraloids	P 5000	5a-pregnan-3a,17,20B-triol
Steraloids	P 5450	5a-pregnan-3a,17,21-triol-20-one
Steraloids	P 3830	5a-pregnan-3B-ol-20-one
Steraloids	P 2490	5a-pregnan-3B,17-diol-20-one
Steraloids	P 2050	5a-pregnan-3B,20a-diol
Steraloids	P 2100	5a-pregnan-3B,20B-diol
Sigma	P 0773	5a-pregnan-3B,17,20B-triol
Steraloids	P 5500	5a-pregnan-3B,17,21-triol-20-one
Steraloids	P 4850	5a-pregnan-3B,11B,17,21-tetrol-20-one
		5B-pregnan free (21 C)
Steraloids	P 7150	5B-pregnan-3,20-dione
Steraloids	P 6300	5B-pregnan-17,21-diol-3,20-dione
Steraloids	P 8090	5B-pregnan-17a-ol-3,20-dione
Steraloids	P 9650	5B-pregnan-11B,17,21-triol-3,20-dione
Steraloids	P 6050	5B-pregnan-3a,20B-diol
Steraloids	P 6570	5B-pregnan-3a,17-diol-20-one
Steraloids	P 8990	5B-pregnan-3a,17a,20a,21-tetrol
Sigma	P 8629	5B-pregnan-3a,17a,20a-triol
Sigma	P 8258	5B-pregnan-3a,17a,20B-triol
Steraloids	P 9000	5B-pregnan-3a,17,20B,21-tetrol
Steraloids	P 9050	5B-pregnan-3a,11B,17,21-tetrol-20-one
Steraloids	P 8540	5B-pregnan-3a,11B,17a,20a,21-pentol

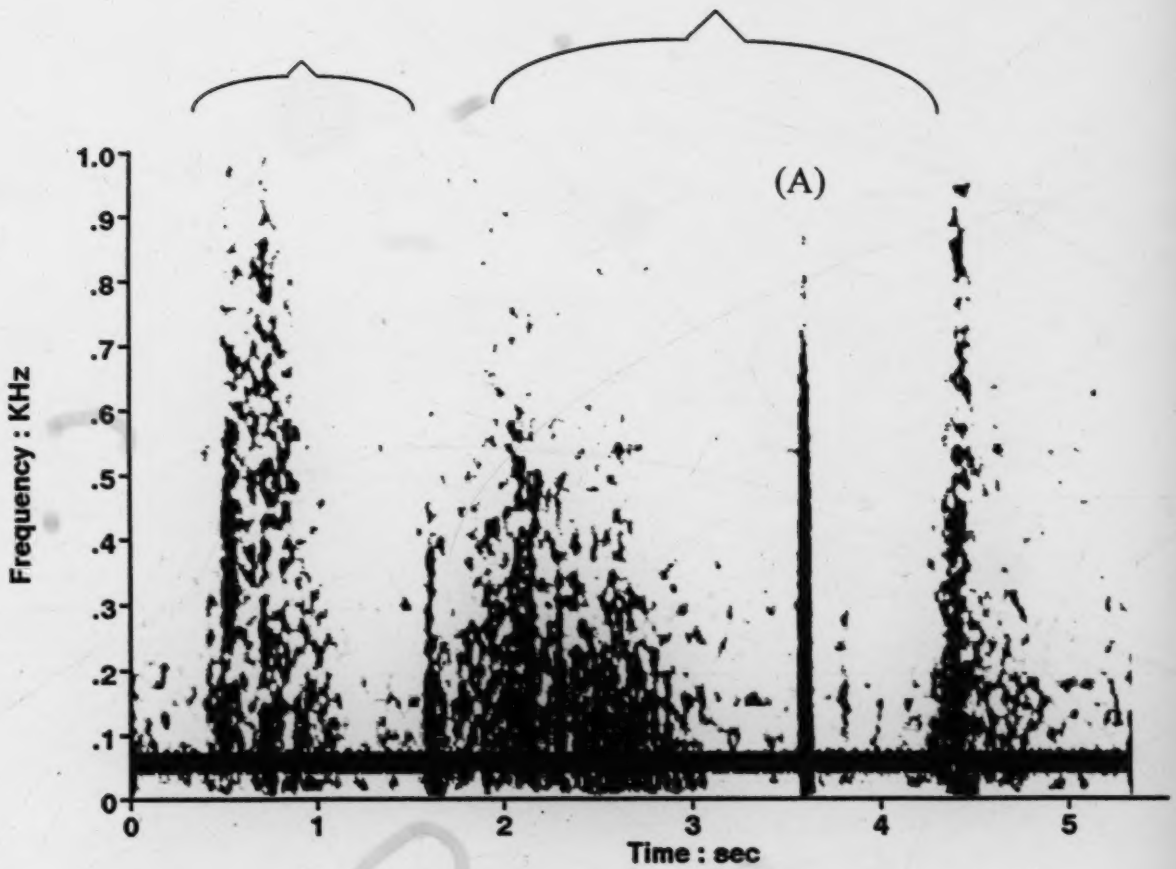
Supplier	Cat #	Chemical name
Steraloids	P 8590	5B-pregnan-3a,11B,17a,20B,21-pentol
Steraloids	P 9200	5B-pregnan-3a,17,20B,21-tetrol-11-one
Steraloids	P 9550	5B-pregnan-3a,17,21-triol-11,20-dione
Steraloids	Q 200	5B-pregnan-3a,17,21-triol-20-one
Steraloids	P6140	5B-pregnan-3B,20B-diol
Steraloids	P6810	5B-pregnan-3B,17-diol-20-one
Steraloids	Q 250	5B-pregnan-3B,17,21-triol-20-one
Sigma	P 1648	5B-pregnan-3B,11B,17,21-tetrol-20-one
		5B-pregnan-gluc
Lambert		5B-pregnan-3a,17a-diol-20-one-3a-gluc
		4-pregnen free (21 C)
Sigma	P 0130	4-pregnen-3,20-dione
Sigma	H 5502	4-pregnen-11a-ol-3,20-dione
Steraloids	Q 3270	4-pregnen-11B-ol-3,20-dione
Sigma	H 5752	4-pregnen-17a-ol-3,20-dione
Sigma	P 6288	4-pregnen-20B-ol-3-one
		4-pregnen-20a-ol-3-one
Sigma	P 6285	4-pregnen-17a,20B-diol-3-one
Sigma	P 6160	4-pregnen-17a,20a-diol-3-one
Sigma	R 0500	4-pregnen-17,21-diol-3,20-dione
Steraloids	Q 1970	4-pregnen-20B,21-diol-3-one
Sigma	D 6875	4-pregnen-21-ol-3,20-dione
Steraloids	Q 4080	4-pregnen-17,20B,21-triol-3-one
Sigma	P 9647	4-pregnen-17,20a,21-triol-3-one
Steraloids	Q 3880	4-pregnen-11B,17,21-triol-3,20-dione
Sigma	P 9129	4-pregnen-17,20B,21-triol-3,11-dione
Steraloids	Q 1550	4-pregnen-11B,21-diol-3,20-dione
Steraloids	Q 1580	4-pregnen-16a,17B-diol-3,20-dione
Steraloids	Q 3920	4-pregnen-14a,17B,21-triol-3,20-dione
Steraloids	Q 4160	4-pregnen-3,11,20-trione
Steraloids	Q 2500	4-pregnen-17,21-diol-3,11,20-trione
Steraloids	Q 3790	4-pregnen-11B,17,20B,21-tetrol-3-one
		4-pregnen-11a,17,21-triol-3,20-dione
Sigma	P 9521	4-pregnen-11B,17a-diol-3,20-dione
		4-pregnen-gluc
Steraloids	Q 1865	4-pregnen-17a,20B-diol-3,20-dione-20-gluc
Steraloids	Q 3740	4-pregnen-21-ol-3,20-dione-21-gluc
Steraloids	Q 3890	4-pregnen-11B,17,21-triol-3,20-dione-21-gluc

Supplier	Cat #	Chemical name
		4-pregnen-SO4
Scott		4-pregnen-20B-ol-3-one-20-SO4
Scott		4-pregnen-17a,20B-diol-3-one-20B-SO4
Scott		4-pregnen-17a,20a-diol-3-one-20a-SO4
Scott		4-pregnen-17,21-diol-3,20-dione-21-SO4
Scott		4-pregnen-17,20B,21-triol-3-one-21-SO4
Sigma	H 5141	4-pregnen-11B,17,21-triol-3,20-dione-xx-SO4
Sigma	C 6162	4-pregnen-11B-21-diol-3,20-dione-21-SO4
		4-pregnen phosphate
		4-pregnen-17a,20B-diol-3,20-dione-20B-PO4
		5-pregnen free
Steraloids	Q 4780	5-pregnen-3B,21-diol-20-one
Steraloids	Q 5900	5-pregnen-3B,17,20B-triol
Steraloids	Q 6050	5-pregnen-3B,17,21-triol-20-one
Sigma	P 9129	5-pregnen-3B-ol-20-one
Sigma	H 5002	5-pregnen-3B,17-diol-20-one
		5-pregnen-gluc
Steraloids	Q 5520	5-pregnen-3B-ol-20-one-3-gluc
		5-pregnen-SO4
Steraloids	Q 4767	5-pregnen-3B,17-diol-20-one-3-SO4
Steraloids	Q 4800	5-pregnen-3B,21-diol-20-one-21-SO4
Steraloids	Q 5545	5-pregnen-3B-ol-20-one-3-SO4

PROSTAGLANDINS: (From Cayman Chemical Company)

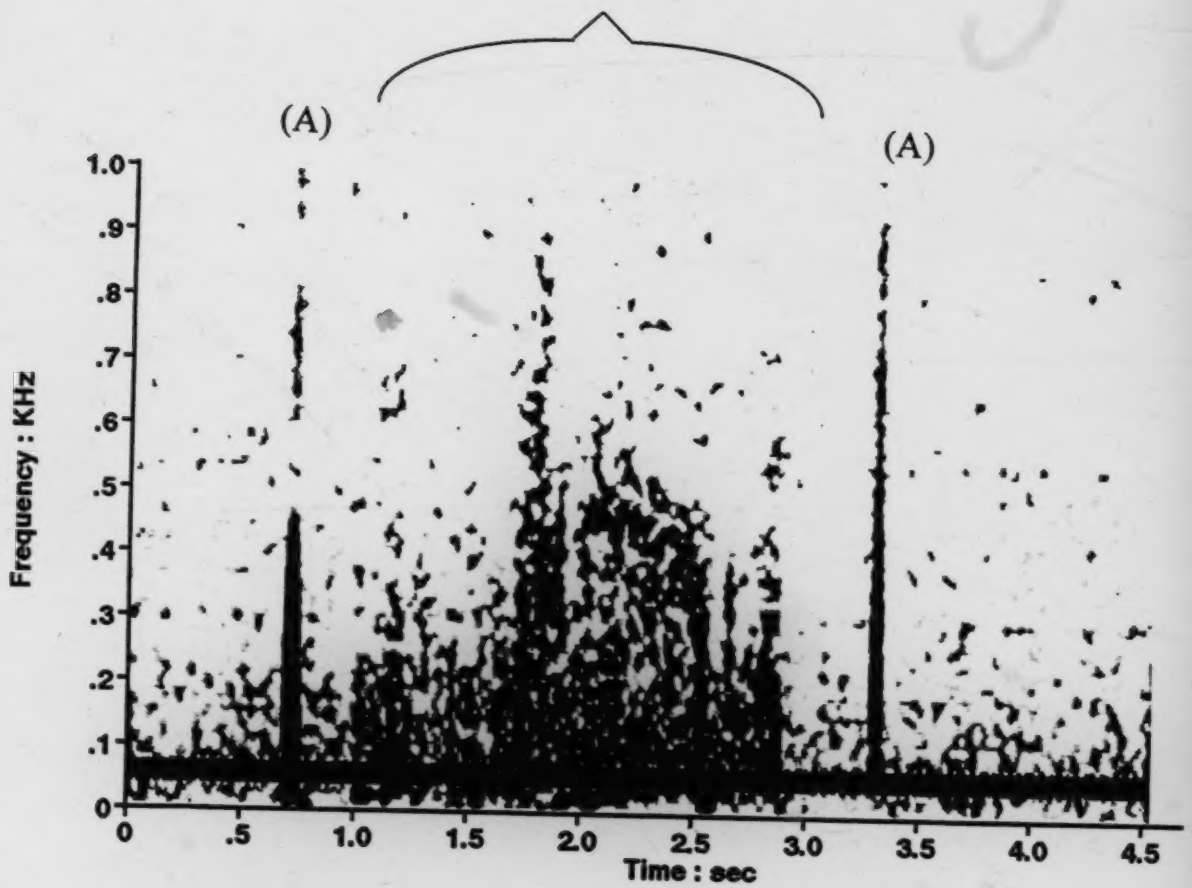
Cat #	Chemical name
13010	Prostaglandin E1
14010	Prostaglandin E2
15010	Prostaglandin F1 alpha
16010	Prostaglandin F2 alpha
16990	Prostaglandin F3 alpha
14720	15-keto Prostaglandin E2
16720	15-keto Prostaglandin F2 alpha
16670	13,14,dihydro-15-keto Prostaglandin F2 alpha

APPENDIX B Spectrograph of gaping behaviour



Recordings were made with a Uher 400 Report-L tape recorder using Ampex tapes and a hydrophone (ITC 6050) which was placed in an aquarium containing single fish. Calls were analyzed using SIGNAL/RTS sound analysis package (Engineering Design, Belmont, Mass.). Each call was sampled at a rate of 5 KHz over the frequency range 0-2 KHz. Sound spectrographs were calculated from 512-point Fast Fourier Transforms (FFTs), with a corresponding frequency bandwidth of 9.8 Hz. Spectrograph shows two gaping behaviours in sequence. Spike at 3.75 sec is an artifact (A) of the recording.

APPENDIX C Spectrograph of coughing behaviour

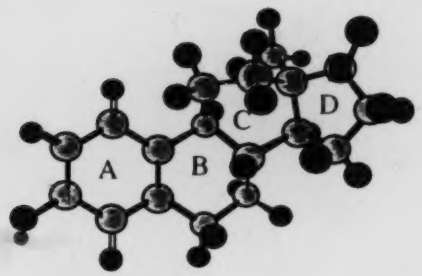


Recordings were made with a Uher 400 Report-L tape recorder using Ampex tapes and a hydrophone (ITC 6050) which was placed in an aquarium containing single fish. Calls were analyzed using SIGNAL/RTS sound analysis package (Engineering Design, Belmont, Mass.). Each call was sampled at a rate of 5 KHz over the frequency range 0-2 KHz. Sound spectrographs were calculated from 512-point Fast Fourier Transforms (FFTs), with a corresponding frequency bandwidth of 9.8 Hz. Spectrograph shows coughing behaviour. Spikes at 0.6 and 3.4 secs are artifacts (A) of the recording.

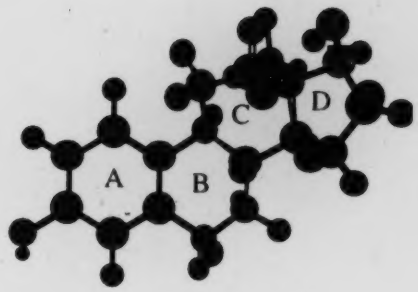
APPENDIX D

E1 receptor

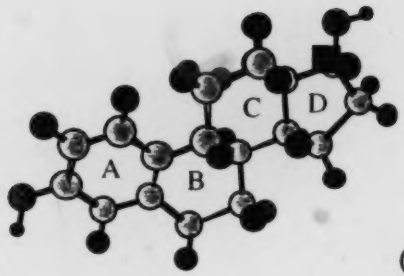
Detected:



Estrone



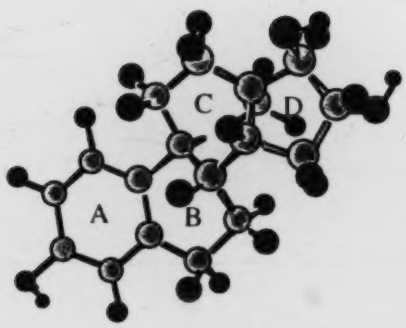
17B-estradiol



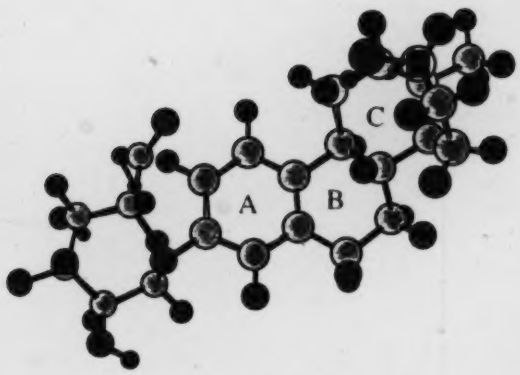
17a-estradiol

Carbon
Oxygen
Hydrogen

Not Detected:



1,3,5 (10)-estratrien-3a, 16a, 17B-triol

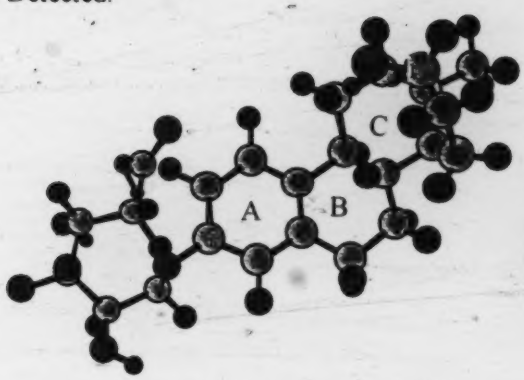


17B-estradiol-3-glucuronide

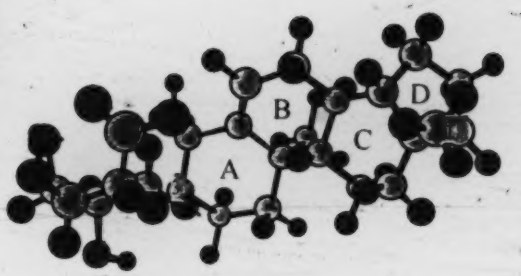
APPENDIX D

E2-3g receptor

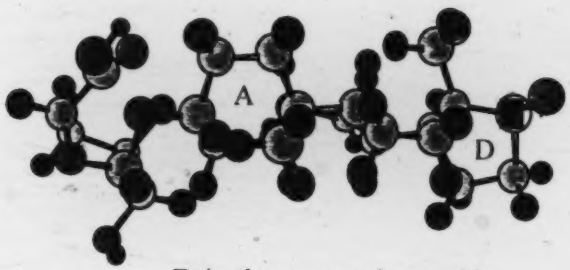
Detected:



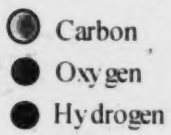
17B-estradiol-3-glucuronide



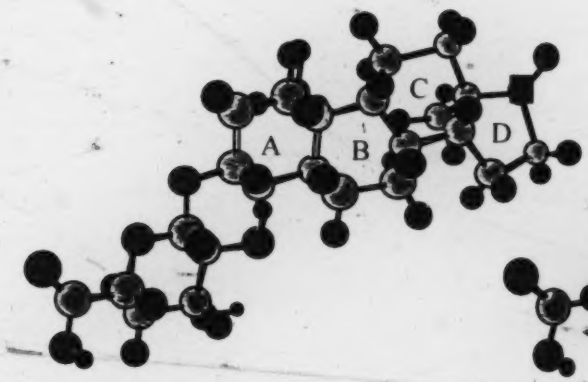
DHEA-g



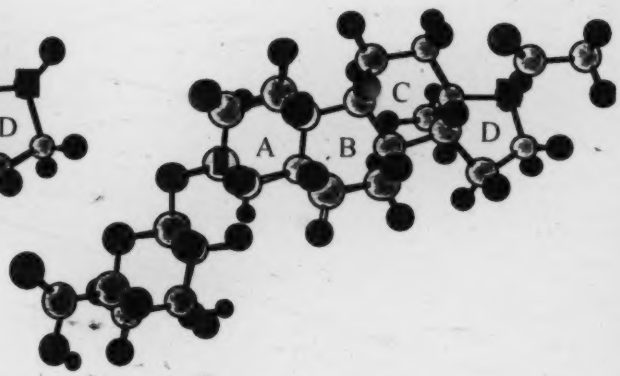
Epiandrosterone-glucuronide



Not detected:



Etiocholanolone-glucuronide

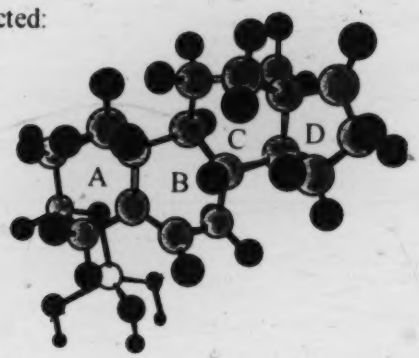


5B-pregnan-3a, 17a-diol-20-one-3a-glucuronide

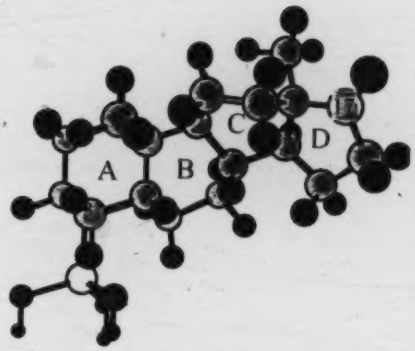
APPENDIX D

DHEA-s receptor

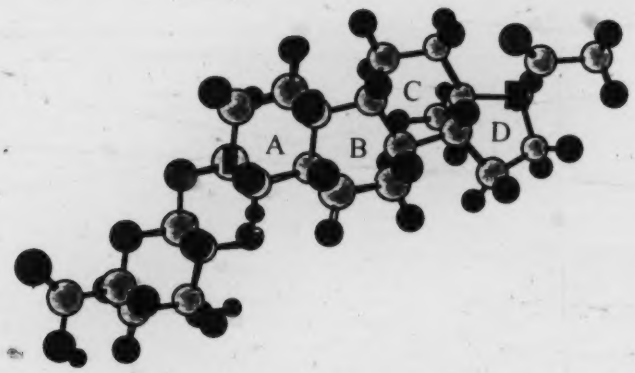
Detected:



DHEA-s



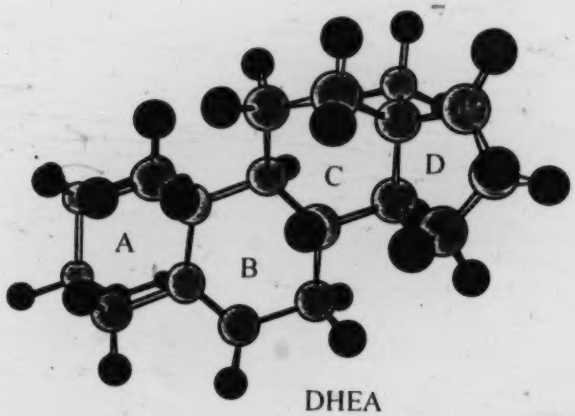
Androsterone-sulphate



Carbon
Oxygen
Hydrogen
Sulphur

5B-pregnan-3a, 17a-diol-20-one-3a-glucuronide

Not detected:

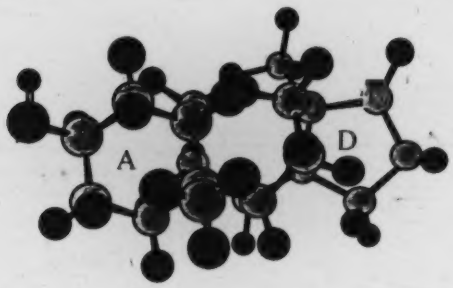


DHEA

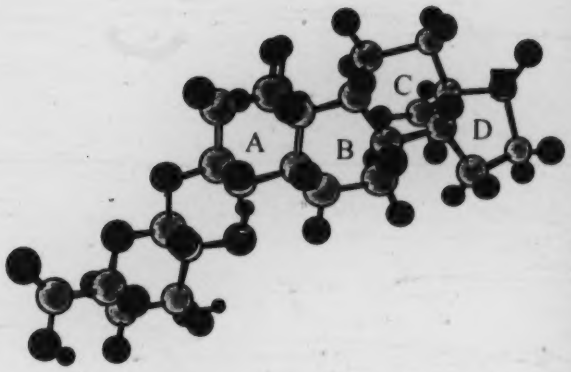
APPENDIX D

Etio receptor

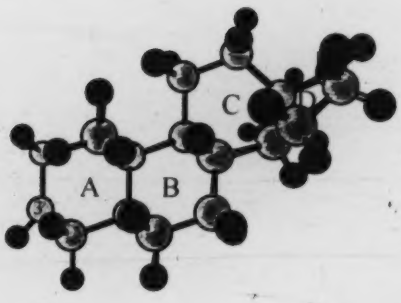
Detected:



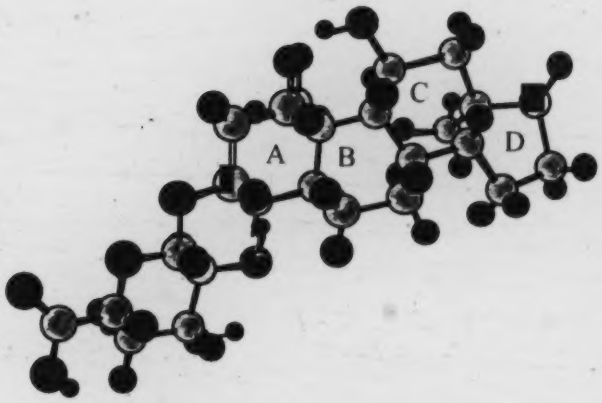
Etiocholanolone



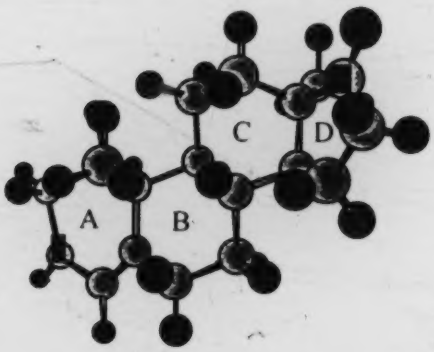
Etiocholanolone-glucuronide



Etiocholane-3,17-dione



11B-Hydroxyetiocholanolone-glucuronide

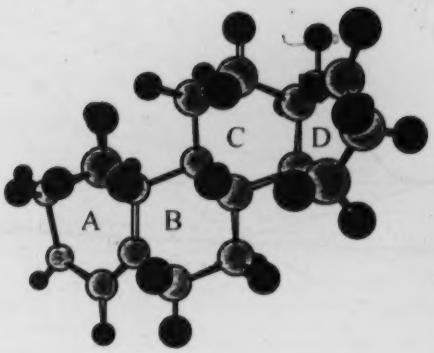


Androstenedione

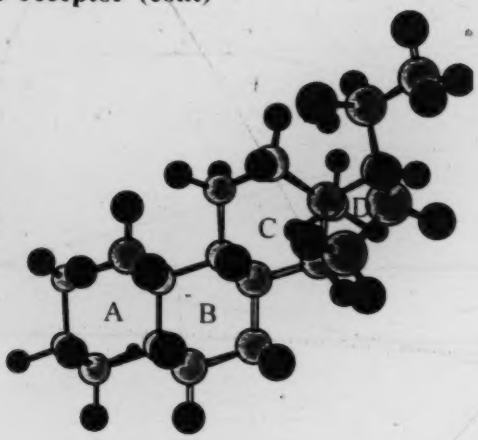
Carbon
Oxygen
Hydrogen

APPENDIX D

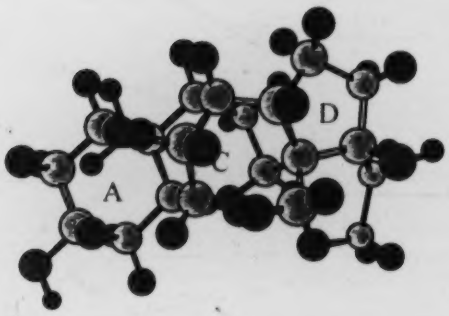
Etio receptor (cont)



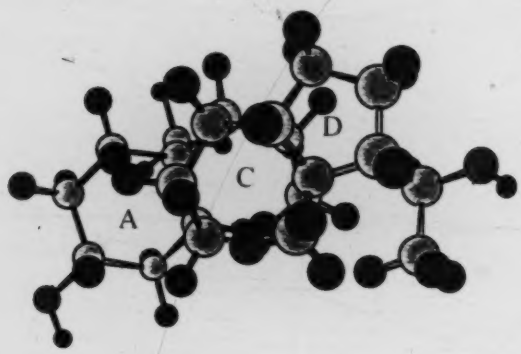
5B-pregnan-3,20-dione



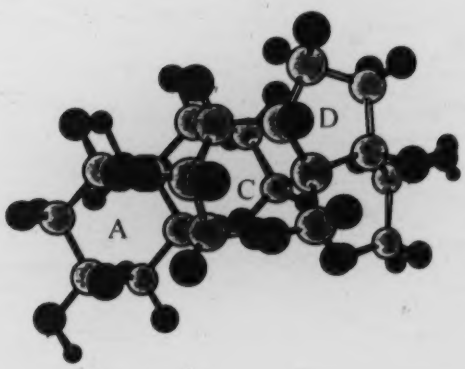
5B-pregnan-3B,20B-diol



5B-pregnan-3a,17-diol-20-one



5B-pregnan-3a,20B-diol



5B-pregnan-3a,17a,20B-triol

Carbon
Oxygen
Hydrogen